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CARDIAC TROPONIN SPECIFIC AUTOANTIBODIES:

Analytical Tools for Exploring Their Impact
on Cardiac Troponin I Testing

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals (**I-IV**):

- I** Savukoski T, Engström E, Engblom J, Ristiniemi N, Wittfooth S, Lindahl B, Eggers KM, Venge P and Pettersson K (2012). Troponin-specific autoantibody interference in different cardiac troponin I assay configurations. *Clin Chem* **58**:1040–1048.

- II** Savukoski T, Twarda A, Hellberg S, Ristiniemi N, Wittfooth S, Sinisalo J and Pettersson K (2013). Epitope specificity and IgG subclass distribution of autoantibodies to cardiac troponin. *Clin Chem* **59**:512–518.

- III** Savukoski T, Ilva T, Lund J, Porela P, Ristiniemi N, Wittfooth S and Pettersson K (2014). Autoantibody prevalence with an improved immunoassay for detecting cardiac troponin-specific autoantibodies. *Clin Chem Lab Med* **52**:273–279.

- IV** Savukoski T, Jacobino J, Laitinen P, Lindahl B, Venge P, Ristiniemi N, Wittfooth S and Pettersson K (2014). Novel sensitive cardiac troponin I immunoassay free from troponin I-specific autoantibody interference. *Clin Chem Lab Med* **52**:1041–1048.

In addition, some unpublished data are included.

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ABBREVIATIONS

aa	amino acids
ACC	American College of Cardiology
ACS	acute coronary syndrome
AHA	American Heart Association
AST	aspartate aminotransferase
BNP	brain natriuretic peptide
BSA	bovine serum albumin
CABG	coronary artery bypass graft
CAD	coronary artery disease
CARMAGUE	Cardiac Marker Guideline Uptake in Europe
CK	creatinine kinase
CK-MB	creatinine kinase muscle-brain fraction
cTn	cardiac troponin
cTnAAb	cardiac troponin specific autoantibody
CV	coefficient of variation
DBUT	Department of Biotechnology, University of Turku
DCM	dilated cardiomyopathy
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESC	European Society of Cardiology
Fab	antigen-binding fragment
FDA	Food and Drug Administration
FRISC	Fragmin and Fast Revascularization during Instability in Coronary artery disease
GUSTO	Global Utilization of Strategies to Open Occluded Arteries
HAAA	human anti-animal antibody
HBV	hepatitis B virus
HCV	hepatitis C virus
ICM	ischemic cardiomyopathy
ID	inhibitory domain
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
ILII	Insulating Layer II buffer solution
IQR	interquartile range
ISFC	International Society and Federation of Cardiology
ITC	cardiac troponin complex
KBS	Kaivogen buffer solution
LBBS	left bundle branch block
LDH	lactate dehydrogenase
LoB	limit of blank
LoD	limit of detection

Abbreviations

LR	low recovery
Mab	monoclonal antibody
MI	myocardial infarction
MR	medium recovery
NA	data not available
N/A	not applicable
NACB	National Academy of Clinical Biochemistry
NIST	National Institute of Standards and Technology
NORIP	Nordic Reference Interval Project
NR	normal recovery
NSTE-ACS	non-ST-segment elevation acute coronary syndrome
NSTEMI	non-ST-segment elevation myocardial infarction
OD	optical density
PCI	percutaneous coronary intervention
PD-1	programmed cell death-1
RA	rheumatoid arthritis
RD	regulatory domain
RP-LC	reversed-phase liquid chromatography
RU	resonance units
RVU	relative value units
SA	streptavidin
SD	standard deviation
SH	sulphydryl
skTn	skeletal troponin
S/LC	signal-to-low control response
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
SRM	standard reference material
STEMI	ST-segment elevation myocardial infarction
Tm	tropomyosin
Tn	troponin
TR-FIA	time-resolved fluoroimmunoassay
TSA	Tris-buffered saline with azide
UA	unstable angina
US	United States
WHF	World Heart Federation
WHO	World Health Organization

ABSTRACT

Cardiac troponin (cTn) I and T are the recommended biomarkers for the diagnosis and risk stratification of patients with suspected acute coronary syndrome (ACS), a major cause of cardiovascular death and disability worldwide. It has recently been demonstrated that cTn-specific autoantibodies (cTnAAb) can negatively interfere with cTnI detection by immunoassays to the extent that cTnAAb-positive patients may be falsely designated as cTnI-negative. The aim of this thesis was to develop and optimize immunoassays for the detection of both cTnI and cTnAAb, which would eventually enable exploring the clinical impact of these autoantibodies on cTnI testing and subsequent patient management.

The extent of cTnAAb interference in different cTnI assay configurations and the molecular characteristics of cTnAAb were investigated in publications **I** and **II**, respectively. The findings showed that cTnI midfragment targeting immunoassays used predominantly in clinical practice are affected by cTnAAb interference which can be circumvented by using a novel 3+1-type assay design with three capture antibodies against the N-terminus, midfragment and C-terminus and one tracer antibody against the C-terminus. The use of this assay configuration was further supported by the epitope specificity study, which showed that although the midfragment is most commonly targeted by cTnAAb, the interference basically encompasses the whole molecule, and there may be remarkable individual variation at the affected sites. In publications **III** and **IV**, all the data obtained in previous studies were utilized to develop an improved version of an existing cTnAAb assay and a sensitive cTnI assay free of this specific analytical interference.

The results of the thesis showed that approximately one in 10 patients with suspected ACS have detectable amounts of cTnAAb in their circulation and that cTnAAb can inhibit cTnI determination when targeted against the binding sites of assay antibodies used in its immunological detection. In the light of these observations, the risk of clinical misclassification caused by the presence of cTnAAb remains a valid and reasonable concern. Because the titers, affinities and epitope specificities of cTnAAb and the concentration of endogenous cTnI determine the final effect of circulating cTnAAb, appropriately sized studies on their clinical significance are warranted. The new cTnI and cTnAAb assays could serve as analytical tools for establishing the impact of cTnAAb on cTnI testing and also for unraveling the etiology of cTn-related autoimmune responses.

TIIVISTELMÄ

Akuutti sepelvaltimotautikohtaus (ACS, *engl.* acute coronary syndrome) on maailmanlaajuisesti merkittävä kuolleisuuden ja työkyvyttömyyden aiheuttaja. Sydänperäiset troponiinit (cTn, *engl.* cardiac troponin) I ja T ovat biomarkkereita, joita suositellaan ACS-potilaiden diagnosointiin ja riskiarviointiin. Hiljattain on kuitenkin osoitettu, että cTn:a tunnistavat autovasta-aineet (cTnAAb, *engl.* cTn-specific autoantibody) voivat negatiivisesti häiritä cTnI:n detektioon käytettäviä immunomäärityksiä; jopa siinä määrin, että cTnAAb-positiiviset potilaat voidaan virheellisesti luokitella cTnI-negatiivisiksi. Väitöstutkimuksen tavoitteena oli kehittää ja optimoida cTnI- ja cTnAAb-määrityksiä, jotka lopulta mahdollistaisivat autovasta-aineiden kliinisen merkityksen arvioimisen.

Ensimmäisessä julkaisussa tutkittiin cTnAAb-häiriön suuruutta erilaisissa cTnI-määrityskonfiguraatioissa ja toisessa cTnAAb:iden molekulaarisia ominaisuuksia. Havainnot osoittivat, että cTnI-molekyylin keskiosan tunnistavat immunomääritykset, joita kliinisessä käytössä olevat määritykset pääasiassa ovat, kärsivät cTnAAb-häiriöstä, ja että häiriö voidaan välttää uudentyypisellä 3+1-määrityksellä. Tässä määrityksessä hyödynnetään kolmea cTnI:n N-terminukseen, keskiosaan ja C-terminukseen sitoutuvaa sitojavasta-ainetta ja yhtä C-terminukseen sitoutuvaa leimavasta-ainetta. Tällaisen määrityskonfiguraation käyttöä tuki myös cTnAAb:iden epitooppikartoitus, jonka perusteella cTnAAb-häiriö kattaa koko cTnI-molekyylin, vaikka autovasta-aineet tavallisimmin sitoutuvat cTnI:n keskiosaan. Lisäksi tulokset osoittivat, että cTnAAb:iden spesifisyydessä voi olla merkittävää yksilöllistä vaihtelua. Tietojen perustella kolmannessa julkaisussa kehitettiin parannettu versio aikaisemmin julkaistusta cTnAAb-määrityksestä ja neljännessä herkkä, cTnAAb-häiriöstä vapaa cTnI-määritys.

Väitöskirjatyön tulokset osoittivat, että ACS-epäillyistä potilaista noin joka 10:nnellä on verenkierrössään havaittavia määriä cTnAAb:ita ja että cTnAAb:t voivat estää cTnI:n määrittämisen, kun ne sitoutuvat samoihin kohtiin cTnI:n immunodetektoon käytettävien vasta-aineiden kanssa. Näin ollen on mahdollista, että cTnAAb-häiriö johtaa tällaisten potilaiden virheelliseen luokitteluun. Koska cTnAAb:iden tiitterit, sitomisvoimakkuudet ja epitooppispesifisyydet sekä endogeenisen cTnI:n pitoisuus yhdessä ratkaisevat cTnAAb:iden lopullisen vaikutuksen, niiden kliinisestä merkityksestä tarvitaan lisätutkimuksia suuremmilla potilasaineistoilla. Kliinistä merkitystä selvitettäessä uudet cTnI- ja cTnAAb-määritykset voivat toimia tärkeinä analyttisinä työkaluina.

1 INTRODUCTION

Acute coronary syndrome (ACS) is a major cause of cardiovascular death and disability worldwide. While timely diagnosis and appropriate therapy are paramount for improving the clinical outcomes of ACS patients, rapid rule-out is equally important for reducing the burden on emergency health care; patients with chest pain and/or other symptoms suggestive of ACS represent a substantial proportion of all acute medical admissions, corresponding to 15-20 million admissions each year in Europe and the United States (US) (Mueller, 2013). However, only one in three patients will be diagnosed with ACS (Mueller, 2013; Conrad and Jarolim, 2014). Therefore, the early identification of ACS in a large and heterogeneous patient population represents one of the greatest diagnostic challenges in emergency medicine.

The determination of circulating cardiac troponin (cTn) I and T along with the evaluation of patient symptoms and electrocardiographic abnormalities currently constitute the cornerstone in the triage of suspected ACS patients (Thygesen *et al.*, 2012a). Since the introduction of the first cTn immunoassays at the end of the 1980s and the beginning of the 1990s, several generations of research and commercial assays have been validated and routinely used. However, only the latest advances in cTn assay technology have enabled the targeted analytical sensitivities and precisions which in turn have enabled the measurement of cTn concentrations in healthy individuals and hence, the detection of ever smaller myocardial injuries. At the same time, the ACS-specificity of cTn testing has decreased and the effect of various confounders has inherently become more significant. While there is now better understanding of the analytical and clinical scenarios influencing cTn results, it has been recognized that some of the former recommendations for the development and clinical use of cTn assays may need re-evaluation.

The following literature review offers an overview of the clinical use of cTns in ACS diagnosis and risk stratification emphasizing the analytical and clinical issues governing cTnI detection. The main focus of the thesis is on circulating cardiac troponin specific autoantibodies (cTnAAb) that have been reported to negatively interfere with cTnI assays by blocking the binding sites of antibodies used in its immunological detection.

2 REVIEW OF THE LITERATURE

2.1 Acute coronary syndrome

ACS refers to any group of clinical symptoms caused by primary myocardial ischemia, i.e. insufficient blood flow to the heart muscle resulting from total or subtotal coronary artery occlusion. It comprises the diagnoses of unstable angina (UA) and myocardial infarction (MI). In UA, the ischemia is not sufficiently severe to cause myocyte necrosis and a diagnostic electrocardiogram (ECG) does not show persistent ST-segment elevation. In MI, the intensity and duration of ischemia is sufficient to cause necrosis and, based on the ECG findings, a patient is diagnosed with either non-ST-segment elevation MI (NSTEMI) or ST-segment elevation MI (STEMI). The Universal Definition of MI, however, is wider and encompasses any amount of necrosis in the setting of ischemia, which for MIs are additionally classified into five subtypes largely according to their pathophysiology (Thygesen *et al.*, 2007; Thygesen *et al.*, 2012a). Of these types, type 1 or spontaneous MI is related to ACS (Pierpont and McFalls, 2009; Newby *et al.*, 2012; Sandoval *et al.*, 2014). Because UA and NSTEMI are both forms of ACS without ST-segment elevation, they can be referred to together as NSTE-ACS. The classification of ACS is summarized in **Figure 1**. (Van de Werf *et al.*, 2008; Hamm *et al.*, 2011; Jneid *et al.*, 2012; O'Gara *et al.*, 2013.)

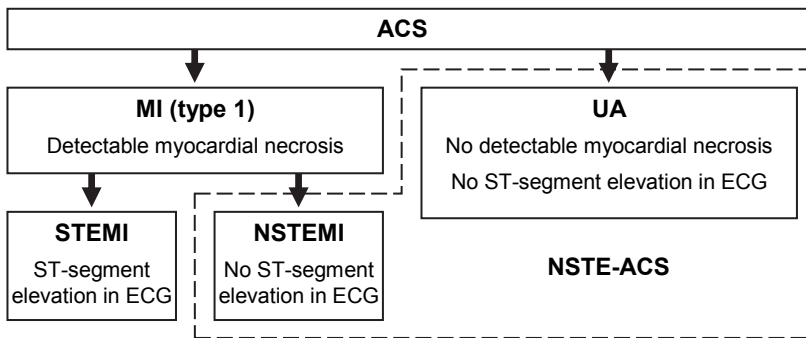


Figure 1. Clinical classification of ACS.

2.1.1 Pathophysiology

The root cause of ACS is coronary atherosclerosis. Atherosclerosis is a complex inflammatory process characterized by a thickening of the arterial wall due to the accumulation of atherosclerotic plaques comprising inflammatory cells, connective-tissue elements, lipids and debris. These plaques contain a necrotic core that is separated from the lumen of the artery by a fibrous cap, which may become thin and vulnerable over time. When such a vulnerable or unstable plaque ruptures, it exposes

its thrombogenic components to the circulation, which promotes platelet aggregation and activates coagulation cascade. Pronounced atherosclerosis of the coronary arteries, i.e. coronary artery disease (CAD), may cause ischemic symptoms when plaques grow large enough to obstruct blood flow to such an extent as to result in myocardial underperfusion or when plaque disruption with resulting intraluminal thrombus blocks the blood flow to the heart. (Gotlieb, 2005; Hansson, 2005; Van de Werf *et al.*, 2008; Hamm *et al.*, 2011; Pant *et al.*, 2013.)

ACS is the result of rupture, fissuring, erosion or dissection of an unstable plaque and subsequent thrombus formation causing partial or complete occlusion of the infarct-related artery, or distal embolization. This etiology differs from that of MI type 2, which is associated with ischemia secondary to either increased oxygen demand or decreased supply, such as in coronary spasm, coronary embolism, anemia, arrhythmias and hypertension. After the onset of ischemia, cell death is not immediate but takes time to develop. Complete necrosis of the cardiomyocytes at risk requires 2-4 hours or longer depending on the presence of collateral circulation to the ischemic zone, persistent or intermittent coronary artery occlusion, the sensitivity of myocytes to ischemia, pre-conditioning, and individual demand for oxygen and nutrients (Alpert *et al.*, 2000; Thygesen *et al.*, 2007). In the minority of ACS patients, the thrombus completely occludes the culprit vessel resulting in STEMI whereas in most of the patients, the thrombus is partially obstructive or only transiently occlusive resulting in NSTEMI-ACS (Yeh *et al.*, 2010). Despite their similar pathogeneses, NSTEMI and UA differ in severity – with no detectable myocyte necrosis, the manifestation of ischemia is labeled UA. However, some experts have hypothesized that with the increased use of extremely sensitive and specific immunoassays for biomarkers of myocardial damage, the diagnosis of UA will eventually disappear eliminating the need for the concept of ACS (Braunwald and Morrow, 2013; Mueller, 2013).

2.1.2 Clinical presentation and diagnosis

Typical symptoms of ACS include various combinations of chest, upper extremity, jaw or epigastric discomfort with exertion or at rest, lasting for at least 20 min. The discomfort is often diffuse with radiation to the left arm. It may be accompanied by other symptoms such as dyspnea, diaphoresis, nausea or syncope. However, these signs are not specific to ACS and can be misdiagnosed as gastrointestinal, neurological, pulmonary or musculoskeletal disorders, for example. Additionally, atypical presentations of ACS are common especially in the elderly, in women and in patients with diabetes (Canto *et al.*, 2002; Culić *et al.*, 2002). As over 50% of patients with severe chest pain do not have ACS and approximately one in three ACS patients do not present with chest pain (Conrad and Jarolim, 2014), the accurate and rapid diagnosis of suspected ACS is essential both for the timely administration of appropriate treatment and for recognizing other cardiac and noncardiac causes of ASC-type symptoms.

The initial patient evaluation includes history and physical examination, 12-lead ECG and measurement of cardiac biomarkers. Despite the fact that a dynamic change in cardiac biomarker values, preferably cTns, is a prerequisite for an MI diagnosis according to the Universal Definition of MI (**Table 1**) (Thygesen *et al.*, 2012a), the role of serial biomarker testing in daily practice is very different for STEMI and NSTEMI as summarized in **Figure 2** (Van de Werf *et al.*, 2008; Hamm *et al.*, 2011; Jneid *et al.*, 2012; O'Gara *et al.*, 2013). STEMI necessitates immediate reperfusion therapy and is diagnosed based on clinical findings and ECG; biomarkers whose concentrations are generally larger than in NSTEMI play a useful prognostic and adjunct confirmatory role but the treatment should not be delayed to wait for the biomarker results. In contrast, NSTEMI diagnosis is based on serial biomarkers; the diagnosis stipulates that a rising and/or falling biomarker with at least one value exceeding the 99th percentile of the healthy reference population is seen in an appropriate clinical setting. As a dynamic change is required to distinguish acute myocardial necrosis from a chronic biomarker elevation, samples should be drawn on admission and 3-6 h later. Additional samples are needed if further ischemic episodes occur or, when the onset of the initial symptoms is unknown. Although the Universal Definition of MI does not provide guidance on the magnitude of the required change, the National Academy of Clinical Biochemistry (NACB) has recommended a relative change of $\geq 20\%$ from the elevated baseline value to be suggestive of an MI (Morrow *et al.*, 2007). However, a rising or falling pattern is not absolutely necessary to meet the diagnosis, if a patient with a high pre-test risk of MI presents late after the onset of symptoms (Thygesen *et al.*, 2012a). If an increased cTn value with or without a dynamic pattern is encountered in the absence of evidence of myocardial ischemia, a careful search for other possible etiologies of cardiac damage should be undertaken.

Table 1. Universal Definition of MI (type 1) (adapted from Thygesen *et al.*, 2012a; Tehrani and Seto, 2013).

Detection of a rise and/or fall of cardiac biomarker values (preferably cTn) with at least one value above the 99th percentile reference limit and with at least one of the following:

- Symptoms of ischemia
 - New ST-segment change or LBBB
 - New pathologic Q wave on ECG
 - Imaging evidence of new loss of viable myocardium or wall motion
 - Identification of an intracoronary thrombus by angiography or autopsy
-

LBBB, left bundle branch block

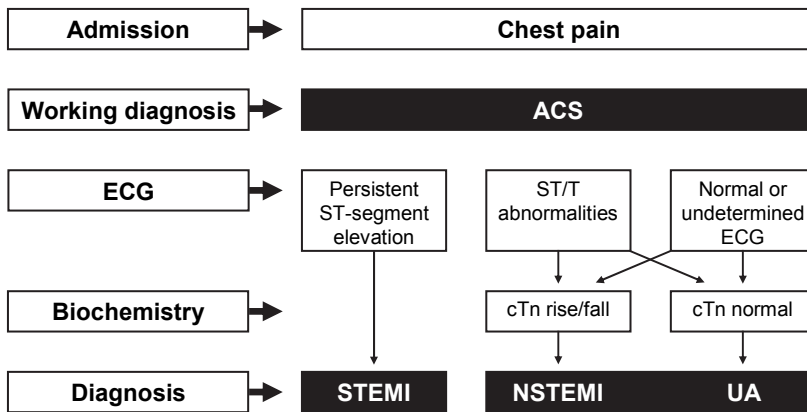


Figure 2. Flowchart for triage and evaluation of a suggestive ACS patient (adapted from Hamm *et al.*, 2011).

2.1.3 Treatment

As a result of improved diagnostic and management strategies, the survival of MI patients has notably improved (Yeh *et al.*, 2010). In STEMI, the cornerstone of acute management is reperfusion therapy with primary percutaneous coronary intervention (PCI) or thrombolysis to limit the extent of cardiomyocyte death by re-establishing normal coronary blood flow in the shortest time possible, which is then complemented with adjunctive medical therapy (e.g. antiplatelet/anticoagulant therapy). In NSTEMI-ACS, appropriate therapy is selected based on risk stratification. All patients should receive conservative pharmacological therapy (e.g. anti-ischemia and antiplatelet/anticoagulant therapy) to provide relief of ischemia and to prevent the recurrence of adverse ischemic events, and high-risk patients should additionally be considered for revascularization. (Van de Werf *et al.*, 2008; Hamm *et al.*, 2011; Jneid *et al.*, 2012; Hanson *et al.*, 2013; O'Gara *et al.*, 2013.)

2.2 Biomarkers of myocardial damage

In the past 60 years, biomarkers of myocardial damage have undergone a progressive evolution (**Table 2**) and the criteria for an MI diagnosis have developed accordingly. The first international guidelines for the diagnosis were established in 1979 by the International Society and Federation of Cardiology (ISFC) and the World Health Organization (WHO) (Bernard *et al.*). In these guidelines, the diagnosis of MI was based on a consensus of two of the following: clinical history, ECG findings and temporal changes in serum enzymes (due to the heterogeneity of clinical symptoms, the fact that the ECG is frequently equivocal, and because biomarkers available at the time were not specific for myocardial injury). The discovery of cTns towards the end of the 1980s and in the beginning of the 1990s (Cummins *et al.*, 1987; Katus *et al.*,

1989), and their subsequent approval for diagnostic use by the US Food and Drug Administration (FDA) changed this situation. Based on evidence obtained with the new assays, NACB first promulgated the need to incorporate blood cTn measurements into MI diagnosis (Wu *et al.*, 1999) and shortly after, a Joint Committee of the European Society of Cardiology (ESC) and the American College of Cardiology (ACC) was convened to re-evaluate the established MI definition (Alpert *et al.*, 2000). As a result, any detectable amount of necrosis caused by ischemia was labeled as MI, and cTns became the recommended biomarkers. These criteria are currently known as the Universal Definition of MI and have been revised twice after expanding the Joint Committee to include representatives from the American Heart Association (AHA) and World Heart Federation (WHF) (Thygesen *et al.*, 2007; Thygesen *et al.*, 2012a).

Table 2. Biomarkers of myocardial damage in chronological order (adapted from Ladenson, 2012; Lewandowski, 2014).

Time	Biomarker	Ultimate destiny
1950s	AST	Obsolete
1960s	Total CK	Obsolete, except when used in conjunction with CK-MB in limited applications
	LDH isoenzymes	Obsolete
1970s	CK-MB isoenzymes, enzyme activity assay	Obsolete, replaced by CK-MB mass immunoassay
1980s	Myoglobin	Never widely used, largely obsoleted after the introduction of cTns
1980s	CK-MB isoenzyme, mass immunoassay	Largely obsoleted after the introduction of cTns, previous gold standard
1990s	cTnI, cTnT	Conventional, sensitive-contemporary and high-sensitivity assays in use

AST, aspartate aminotransferase; CK, creatine kinase; CK-MB, CK muscle-brain fraction; LDH, lactate dehydrogenase

The initial evaluation of suspected ACS patients using cTn measurements not only serves a diagnostic purpose but may also be used for risk stratification and therapy guiding (Morrow *et al.*, 2001; Steg *et al.*, 2009). A high cTn concentration correlates with a more severe degree of CAD and a larger infarct size (Lindahl *et al.*, 2001; Wong *et al.*, 2001; Licka *et al.*, 2002; Steen *et al.*, 2006). Hence, higher cTn levels help identify those ACS patients who are most likely to benefit from more aggressive therapy, such as early invasive procedures.

Although cTn release has excellent specificity for cardiac injury, the introduction of progressively more sensitive cTn assays has demonstrated that myocardial damage is not specific to ACS. These non-ACS related conditions leading to elevated cTn can be divided into causes related to secondary myocardial ischemia (MI type 2), diseases not associated with myocardial ischemia, and conditions where the exact mechanisms are uncertain or multifactorial (see **Table 3**). It is important to understand that because cTn elevations in most of these situations are also associated with an increased risk of an adverse outcome, the detection of elevated cTn levels in the absence of ACS should

prompt a careful search for the underlying cause and when possible, the initiation of cause-specific treatment (Alcalai *et al.*, 2007; Kavsak *et al.*, 2012; Wang *et al.*, 2012b). Differentiating between ACS and non-ACS events is challenging. In particular, diagnosing MI type 2 in patients presenting to an emergency department has caused confusion among clinicians; the same diagnostic criteria as for MI type 1 should be used for patient evaluation but little is known about the best treatment strategies (Pierpont and McFalls, 2009; Newby *et al.*, 2012; Giannitsis and Katus, 2013; Alpert *et al.*, 2014; Sandoval *et al.*, 2014). Thus, better protocols for clarifying the etiology of cTn releases are warranted while in the meantime, it remains important to interpret laboratory data in the clinical context in which they are found. (Agzew, 2009; Thygesen *et al.*, 2010; Giannitsis and Katus, 2013; Marini *et al.*, 2013.)

Table 3. Different causes of elevated cTn (adapted from Thygesen *et al.*, 2010; Thygesen *et al.*, 2012a; Tehrani and Seto, 2013).

Damage related to primary myocardial ischemia (MI type 1)
<ul style="list-style-type: none"> • Rupture of an atherosclerotic plaque • Intraluminal coronary artery thrombus formation
Damage related to secondary myocardial ischemia (MI type 2)
<ul style="list-style-type: none"> • Tachy- or bradyarrhythmias • Aortic dissection or severe aortic valve disease • Hypertrophic cardiomyopathy • Severe respiratory failure • Severe anemia • Hypertension • Coronary spasm • Coronary embolism or vasculitis • Coronary endothelial dysfunction without significant CAD
Damage not related to myocardial ischemia
<ul style="list-style-type: none"> • Cardiac contusion • Cardiac incisions • Pacing or defibrillator shocks • Rhabdomyolysis with cardiac involvement • Myocarditis • Cardiotoxic agents, e.g. anthracyclines
Multifactorial or indeterminate myocardial damage
<ul style="list-style-type: none"> • Heart failure • Stress cardiomyopathy • Severe pulmonary embolism or pulmonary hypertension • Sepsis and critically ill patients • Renal failure • Severe acute neurological diseases, e.g. stroke • Infiltrative diseases, e.g. amyloidosis • Extreme exertion

2.3 Cardiac troponin I

The contractile apparatus of skeletal and cardiac muscle cells contains thin actin filaments and thick myosin filaments organized in sarcomeres. The sliding of these filaments along each other forms the molecular basis for striated muscle contraction, and is regulated by a troponin (Tn) complex and tropomyosin (Tm). The ternary Tn complex, which is attached periodically along the thin filament, is composed of three different subunits with specific functions: inhibitory TnI, Tm-binding TnT and Ca^{2+} -binding TnC. Upon muscle contraction, electrical stimulation increases the intracellular Ca^{2+} concentration. This leads to complex conformational changes in the Tn structure followed by the movement of TnI and Tm on the thin filament, which eventually enables the adenosine triphosphate driven interaction between actin and myosin, and subsequent development of muscle contraction. When Ca^{2+} is pumped back into the sarcoplasmic reticulum, the conformational changes are reverted and the muscle relaxes. TnI and TnT, but not TnC, exist as unique, recognizable isoforms in myocardium. Thus their cardiac forms, cTnI and cTnT, found in the circulation can serve as highly specific and sensitive indicators of recent or persistent cardiomyocyte damage. The diagnostic utilities of cTnI and cTnT are comparable but due to the tight patent restrictions, cTnT assays are commercially available from only two manufacturers (Roche Diagnostics and Radiometer). Although many of the analytical and clinical issues governing cTnI detection apply also to cTnT, the cTnT molecule and its determination were not included in this thesis. (Bandman, 1992; Farah and Reinach, 1995; Katrukha, 2013.)

2.3.1 Expression and structure

Human TnI is expressed in three isoforms encoded by separate genes: slow- and fast-twitch skeletal troponin (skTn) I, and cTnI (Wade *et al.*, 1990; Bhavsar *et al.*, 1996; Tiso *et al.*, 1997). Compared to the skeletal isoforms, cTnI exhibits approximately 40% sequence homology and is larger (209 amino acids, aa, 24 kDa) due to an additional 30-membered N-terminal peptide (Bhavsar *et al.*, 1996; Apple *et al.*, 2012a). Slow-twitch skTnI is predominantly expressed during embryonic and fetal development of the human heart and completely substituted with cTnI by the ninth month after birth (Saggin *et al.*, 1989; Bhavsar *et al.*, 1991; Sasse *et al.*, 1993), while cTnI is expressed in neither normal nor diseased skeletal muscle (Bodor *et al.*, 1995; Sacks, 1999).

The cTnI molecule consists of five domains presented in **Figure 3**. The cardiac-specific N-terminal domain (aa 2-32) includes an acidic part, a proline helix and a part carrying two serine residues in positions 23 and 24 (Holroyde *et al.*, 1979; Mittmann *et al.*, 1990; Ferrières *et al.*, 2000; Howarth *et al.*, 2007). The phosphorylation of these serine residues decreases the Ca^{2+} sensitivity of cardiomyocytes, presumably by decreasing the affinity of cTnI to TnC (al-Hillawi *et al.*, 1995; Howarth *et al.*, 2007). The IT-arm (aa 42-136) is composed of two α -helices (H1 and H2) connected by a short linker (Takeda *et al.*, 2003). It is the least flexible part of the cTnI molecule and

has a structural role proving contact with both TnC and cTnT, and orientating cTnI in the Tn complex. The structure of the inhibitory domain (ID) (aa 137-148) has not been definitely resolved probably due to its flexible nature. This domain binds to actin in the absence of Ca^{2+} preventing actin-myosin interactions in unstimulated muscle cells (Farah *et al.*, 1994; Dong *et al.*, 2003; Takeda *et al.*, 2003; Kobayashi *et al.*, 2009). The regulatory domain (RD) (aa 149-160) contains a short α -helix (H3) and interacts with TnC at high Ca^{2+} concentrations (Li *et al.*, 1999; Wang *et al.*, 2002). This interaction with Ca^{2+} -saturated TnC dissociates the mobile domain (aa 163-210), which includes an α -helix (H4) and an unresolved C-terminal part (Takeda *et al.*, 2003; Galinska-Rakoczy *et al.*, 2008; Galinska *et al.*, 2010; Wang *et al.*, 2012a), from actin upon stimulation and, therefore, serves as a Ca^{2+} -sensitive molecular switch in muscle contraction (Farah *et al.*, 1994; Perry, 1999). The models proposed for the Tn complex function and regulation have been reviewed elsewhere in detail (Katrukha, 2013; Sheng and Jin, 2014).

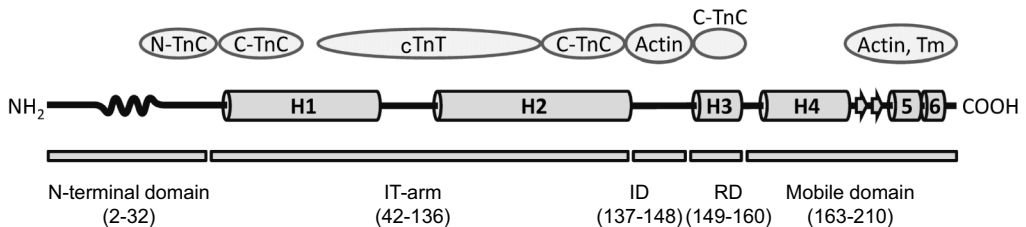


Figure 3. A schematic presentation of the cTnI molecule (Katrukha, 2013). The bars represent the five cTnI domains, whose secondary structures are described in the middle. The wavy curve in the secondary structure symbolizes a proline helix whereas barrels and short arrows represent α -helices and β -strands, respectively. Interaction sites with the other molecules of the contractile apparatus are indicated with ovals. Republished with permission of Consultants Bureau Enterprises; permission conveyed through Copyright Clearance Center, Inc.

2.3.2 Release characteristics and forms in circulation

The vast majority of cTnI is bound to the thin filaments of myocytes while only a minor fraction, around 2% to 8%, exist free in the early-releasable cytosolic pool, eventually representing a precursor pool of sarcomere assembly (Adams *et al.*, 1994; Bleier *et al.*, 1998; Higgins and Higgins, 2003; Agewall and Giannitsis, 2014). Although the mechanisms by which cTnI is released into the circulation are not fully understood, the prevailing view is that cTnI is only released after irreversible myocardial damage; primarily after necrosis but presumably tiny amounts can also be released by apoptosis and normal cardiomyocyte turnover (**Figure 4**) (Narula *et al.*, 1996; James, 1998; Missov and De Marco, 1999; Anversa *et al.*, 2002; Bergmann *et al.*, 2009; Wu and Christenson, 2013). Consequently, myocyte necrosis causes cell membrane disruption and cTnI leakage into the extracellular space. Via local microvascular and lymphatic drainage, cTnI then enters the systemic circulation where it can be detected in a few hours from the onset of ischemic symptoms. It is assumed

that the initial cTnI increase originates from the rapid release of the cytoplasmic fraction, which is subsequently followed by the gradual breakdown of myofibrils. The latter is believed to explain the prolonged presence of cTnI in blood (4-7 days) despite the presumably short half-life of the protein (hours) (Morrow *et al.*, 2007; Dunn *et al.*, 2011).

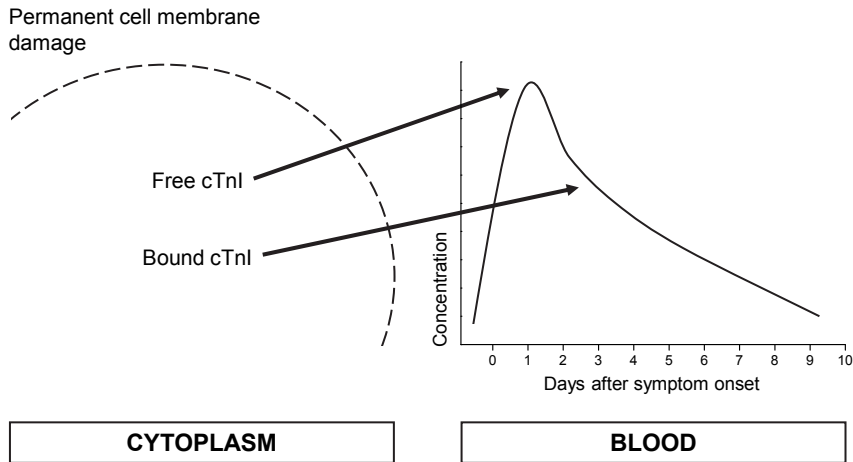


Figure 4. Irreversible myocardial damage and cTnI release (adapted from Hickman *et al.*, 2010). Loss of cell membrane integrity initially releases the free cTnI in the cytoplasm, which is followed by sustained release due to the gradual degradation of contractile proteins.

Contrary to the hypothesis presented above, with modern assays cTnI can be detected in the majority of MI patients already during the first hours of ischemia and in patients without any subsequent evidence of irreversible myocardial damage e.g. in participants of extreme sports (Vilela *et al.*, 2014). In the latter group, cTnI typically disappears from blood substantially faster than in MI patients, i.e. they lack the sustained release. Therefore, it has been proposed that the cytoplasmic pool can also be released during reversible cardiomyocyte damage (Bergmann *et al.*, 2009; White, 2011; Wu and Christenson, 2013). The proposed release mechanisms include cellular release of proteolytic cTnI degradation products (Gao *et al.*, 1997; Feng *et al.*, 2001; van der Laarse, 2002), increased cellular wall permeability due to myocardial stretch or ischemia (Hessel *et al.*, 2008), and formation and release of membranous blebs (Hickman *et al.*, 2010). Because it is difficult to isolate and ascertain the contribution of micronecrosis and other mechanisms, cTnI in blood does not unequivocally define the underlying mechanism for its release.

In peripheral circulation, cTnI is present both as free and complexed forms. In MI, cTnI has mainly been found in the binary cTnI-TnC complex, with smaller amounts of the ternary cTnI-cTnT-TnC complex and free cTnI (Katrukha *et al.*, 1997; Wu *et al.*, 1998; Giuliani *et al.*, 1999; Labugger *et al.*, 2003). Additionally, cTnI is susceptible to

progressive proteolytic degradation in necrotic myocytes, in the circulation and in collected blood (Katrukha *et al.*, 1998; Morjana, 1998; McDonough *et al.*, 2001; Law *et al.*, 2005). In particular, the N- and C-terminal parts of the molecule are less stable compared to the cTnI midfragment (aa 30-110) protected by TnC binding. Furthermore, the cTnI molecule undergoes various posttranslational modifications. It has been demonstrated that approximately 50% of the cTnI in the blood of MI patients is phosphorylated; particularly serine residues at positions 23 and 24 but there are many additional cTnI phosphorylation sites whose functional roles are not yet thoroughly understood (Katrukha *et al.*, 1999; Labugger *et al.*, 2000; Katrukha, 2013; Sheng and Jin, 2014). Other possible posttranslational modifications of cTnI include oxidation and reduction of two cysteine residues at positions 80 and 97 (Vallins *et al.*, 1990; Katrukha *et al.*, 1999). Hence, a number of different molecular cTnI forms are found both in cardiac tissue and in the circulation. It is suspected that the proportion of the forms may be time dependent and disease-specific (Katrukha *et al.*, 1997; Wu *et al.*, 1998; Labugger *et al.*, 2000; Colantonio *et al.*, 2002; Cobbaert *et al.*, 2008; Hessel *et al.*, 2008). Because the pronounced heterogeneity of cTnI can affect cTnI recognition by assay antibodies selected for its immunological detection, and because our knowledge of the exact nature of these forms is rather limited, the current cTnI assays are designed to recognize all of the different forms equally.

2.4 Immunoassays for cardiac troponin I

The first investigational cTnI immunoassay using polyclonal antibodies was described in 1987 (Cummins *et al.*). Five years later, cTnI-specific monoclonal antibodies (Mab) were developed (Bodor *et al.*, 1992; Larue *et al.*, 1992) closely followed by the release of the first commercial assays by Sanofi Pasteur (Larue *et al.*, 1993) and Dade Behring (Adams *et al.*, 1993). Of these, Dade Behring's automated cTnI assay became widely used in clinical practice. The early cTnI assays aimed at matching the clinical performance of CK-MB. Since then, assays have evolved substantially, and the latest generation of cTnI assays are 200- to 500-fold more sensitive than the investigational and commercial assays initially described in the early 1990's (Panteghini, 2013). This development has been especially propelled by the first Universal Definition of MI (Alpert *et al.*, 2000) which inspired assay manufacturers to improve cTn assays to reach the total precision (coefficient of variation, CV) of 10% at the 99th percentile of a healthy reference population. Lately, the guideline-recommended assay performance has been achieved.

In contrast to conventional assays, the new higher sensitivity cTnI assays have enabled the optimal discrimination between low cTnI levels and analytical noise, and reliable measurement of values in the region of the 99th percentile of a healthy population. Although no universally agreed nomenclature of cTn assays exists, in a scorecard concept an assay needs to meet two basic criteria to be considered high-sensitivity (Apple, 2009). First, the CV at the 99th percentile must be $\leq 10\%$. Second, cTn must be measureable above the assay's limit of detection (LoD) for at least 50% (and ideally 95%) of healthy individuals. The majority of modern assays, i.e. sensitive-

contemporary assays, lack these features; they meet the recommended analytical precision at a higher concentration than the 99th percentile and are typically able to quantitate cTn in <10% of healthy individuals (Apple *et al.*, 2012b). Sensitive-contemporary assays can be categorized into three groups based on the CV of the 99th percentile – not acceptable (>20%), clinically usable (10-20%) and guideline acceptable (<10%). According to the evidence-based data, both clinically usable and guidance acceptable cTn assays can be used in clinical practice without significant misclassification of MI patients (Apple *et al.*, 2005; Kupchak *et al.*, 2006; Jaffe and Apple, 2010). The major drawback of this naming system, however, is that it does not consider the actual clinical performance that may vary between differently configured cTnI assays (Venge and Lindahl, 2013).

A number of manufacturers currently provide immunoassays for cTnI measurement and although most of them follow the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommended quality specifications for cTn immunoassays (Panteghini *et al.*, 2001; Jaffe, 2011; Apple *et al.*, 2012b), the remaining dissimilarities between methods continue to result in noninterchangeable cTnI results. This lack of standardization restricts comparison of cTnI values between laboratories and research reports. Therefore, the assay characteristics including decision limits must be separately determined for each assay before its clinical implementation. The analytical characteristics of commercially available cTnI assays are listed in **Table 4**.

Table 4. Analytical characteristics of commercial cTnI assays declared by the manufacture (adapted from IFCC, 2013).

Company / platform(s) / assay	LoB ^A (ng/L)	LoD ^A (ng/L)	99 th percentile (CV) (ng/L)	10% CV (ng/L)	Epitopes (aa) recognized by capture / tracer antibodies
Abbott AxSYM ADV	20		40 (14.0%)	160	87-91, 41-49 / 24-40
Abbott Architect	<10		28 (14.0%)	32	87-91, 24-40 / 41-49
Abbott Architect STAT hs-cTnI ^B	0.7-1.3	1.1-1.9	26.2 (4.0%)	4.7	24-40 / 41-49
Abbott i-STAT	20		80 (16.5%)	100	41-49, 88-91 / 28-39, 62-78
Alere Triage SOB	50		NA (NA)	NA	NA / 27-40
Alere Triage Cardio 3	2	10	22 (17.0%)	37	27-39 / 83-93, 190-196
Beckman Coulter Access Accu	10		40 (14.0%)	60	41-49 / 24-40
bioMerieux Vidas Ultra	<10	<10	10 (27.7%)	110	41-49, 22-29 / 87-91, TnC-specific
Mitsubishi PATHFAST cTnI ^B		1	20 (5.2%)	3.1	41-49 / 71-116, 163-209
Mitsubishi PATHFAST cTnI-II ^C	2	8	29 (5.0%)	14	41-49 / 71-116, 163-209
Ortho VITROS Troponin I ES	7	12	34 (10.0%)	34	24-40, 41-49 / 87-91
Radiometer AQT90 FLEX TnI		9.5	23 (17.7%)	39	41-49, 190-196 / 137-149
Response Biomedical RAMP	30		100 (20.0%)	210	85-92 / 26-38
Roche E 2010 / cobas e 411 / Roche E 170 / cobas e 601 / 602 cTnI		160	160 ^E (NA)	300	87-91, 190-196 / 23-29, 27-43
Siemens ADVIA Centaur TnI-Ultra	6		40 (8.8%)	30	41-49, 87-91 / 27-40
Siemens Dimension Rxl CTNI	40 ^D		70 (15%-22%)	140	27-32 / 41-56
Siemens Dimension EXL TNI	10	17	56 (10.0%)	50	27-32 / 41-56
Siemens Dimension VISTA CTNI	15		45 (10.0%)	40	27-32 / 41-56
Siemens IMMULITE 1000 Turbo ^B	150		300 (14%)	590	87-91 / 27-40
Siemens IMMULITE 1000 ^B	100		190 (11%)	220	87-91 / 27-40
Siemens IMMULITE 2000 XPI ^B	200		290 (10.3%)	320	87-91 / 27-40
Siemens IMMULITE 1000 Turbo ^C	150		NA (NA)	640	87-91 / 27-40
Siemens Stratus CS cTnI	30 ^D		70 (10.0%)	60	27-32 / 41-56
Tosoh ST AIA-PACK	60		60 ^E (8.5%)	NA	41-49 / 87-91

LoB, limit of blank; NA, data not available; ^A, determined according to CLSI Guideline EP17-A³; ^B, claims are valid for use outside of the US; ^C, claims are valid for use in the US; ^D, analytical sensitivity determined by running 20 replicates of a zero concentration sample; ^E, a 99th percentile concentration equal to an assay's LoD is unlikely to have acceptable imprecision for reliable cTnI measurement

2.4.1 Sensitive-contemporary and high-sensitivity assays

The new generation of sensitive-contemporary and high-sensitivity cTnI assays has enabled the reliable detection of minor cTnI elevations, and thus improved the diagnostic and prognostic accuracy of cTnI assays (Keller *et al.*, 2009; Keller *et al.*, 2011; Mills *et al.*, 2011; Kavsak *et al.*, 2012). The benefits of using sensitive assays instead of their conventional counterparts have been most evident in the early hours after symptom onset and in challenging subgroups such as elderly patients or patients with pre-existing CAD (Reiter *et al.*, 2011; Reiter *et al.*, 2012). It has been demonstrated that the rule-in and rule-out can be established within 2-3 h from symptom onset but even an 1 h approach and the obviation of the need for serial testing in selected patients have been proposed (Keller *et al.*, 2011; Reichlin *et al.*, 2011; Reiter *et al.*, 2011; Than *et al.*, 2012; Cullen *et al.*, 2013). Earlier decision making should result in earlier treatment and/or discharge of patients with consequent improvement of patient outcomes and potentially significant economic impact on acute care. Although both sensitive-contemporary and high-sensitivity cTnI assays are superior to conventional cTnI assays, it remains unknown whether high-sensitivity assays provide a diagnostic advantage over sensitive-contemporary assays (de Lemos, 2013).

The increased sensitivity for detecting myocardial injury, however, is linked with the reduced specificity for identifying ACS. As cTnI can be detected in a greater number of non-ACS patients with acute and chronic conditions, concern regarding the application of higher sensitivity cTnI assays in a population with a lower MI prevalence, in particular, has been voiced (Reichlin *et al.*, 2009; Keller *et al.*, 2011; Gassenmaier *et al.*, 2012; de Lemos, 2013). The lower specificity may result in unnecessary hospital admissions, misdiagnoses, initiation of potentially detrimental therapies and poorer clinical outcomes for patients. In order to maintain the ACS-specificity of the assays, the use of higher cutoff values than the 99th percentiles and adaptation of different algorithms have been proposed (Apple and Morrow, 2012; Gassenmaier *et al.*, 2012; Kavsak *et al.*, 2012; Than *et al.*, 2012; Cullen *et al.*, 2013). Nevertheless, because an increase in cTnI levels is not specific for the etiology of cardiac cell death, the clinician's responsibility in interpreting cTnI results in the clinical context where they are found increases. On the other hand, the increased sensitivity of cTnI assays provides a new tool for studying normal and altered myocardial function and for population screening of subclinical diseases. However, until further data and instructions are available about cTnI testing in non-ACS settings, measuring cTnI levels to diagnose or to determine a prognosis for any of these conditions is not recommended.

Diagnostic algorithms based on cTnI changes, or deltas, have been shown to improve diagnostic specificity of cTnI for the MI diagnosis. Serial testing is especially important for the interpretation of low level positive results, and it has been suggested that the serial changes detected with sensitive cTnI assays in an individual patient may be of greater value than the use of population based reference values (Jaffe, 2006; Wu

and Jaffe, 2008; Apple *et al.*, 2009; Wu *et al.*, 2009; Hickman *et al.*, 2014). It is, however, important to note that also acute cardiac conditions other than MI such as myocarditis, sepsis, heart failure and renal failure may produce substantial temporal cTnI changes. While the NACB-recommended relative change of 20% was empirically selected as a value that would exceed the analytical variation of conventional assays (Morrow *et al.*, 2007), conjoint biological and analytical variation of more sensitive cTnI assays seems to be much higher. In recent reports, the short- and long-term biological variation of cTnI has been in the range of 3%-37% and 3%-117%, respectively, and acceptable specificities and positive predictive values have been obtained with notably higher relative changes ranging from 50% to 250% (Kavsak *et al.*, 2010; Eggers *et al.*, 2011; Keller *et al.*, 2011; Reichlin *et al.*, 2011; Mueller *et al.*, 2012; Thygesen *et al.*, 2012b; Nordenskjöld *et al.*, 2013; Wu and Christenson, 2013). Furthermore, because substantial relative changes are common in low cTnI levels despite small absolute increases, the use of absolute change values has recently been introduced. Compared to the use of relative changes this concept has provided higher diagnostic accuracy, although its implementation has not yet been unequivocally settled (Kavsak *et al.*, 2010; Keller *et al.*, 2011; Reichlin *et al.*, 2011; Haaf *et al.*, 2014). In both relative and absolute delta approaches, the decision limits have to be estimated individually for each assay and for different time windows keeping in mind the intended use (Apple and Morrow, 2012). ESC currently proposed using a relative change of $\geq 20\%$ at 3 h in patients with the initial cTnI value above the 99th percentile and a relative change of $\geq 50\%$ in patients with the initial cTnI value below or close to the 99th percentile (Hamm *et al.*, 2011; Thygesen *et al.*, 2012b). However, these criteria based on such small concentration changes seem small in comparison with the limited data available, and further studies and statistical testing are needed.

2.4.2 Challenges

Although the analytical sensitivities and precisions of currently available cTnI assays have substantially improved in comparison to the previous assay generations, numerous other factors confound the immunological detection of cTnI and the use of cTnI assays in clinical practice. Firstly, cTnI assays just like any other immunoassays are prone to a variety of analyte-independent preanalytical and analytical problems that can lead to the detection of falsely high or low cTnI concentrations (Selby, 1999; Tate and Ward, 2004; Sturgeon and Viljoen, 2011; Lippi *et al.*, 2013). Exogenous errors such as calibration errors, reagent deterioration and analyzer malfunction should be detected by appropriate quality control systems whereas sample-dependent endogenous errors such as hemolysis, fibrin clots and interferences from heterophilic antibodies, rheumatoid factor, human anti-animal antibodies (HAAA) and complement are more difficult to identify. Heterophilic antibodies, rheumatoid factor and HAAA affect almost all immunoassays to some extent and their effect can be minimized by using blocking antibodies, recombinant antibody fragments and humanized antibodies, for example (Selby, 1999; Tate and Ward, 2004; Sturgeon and

Viljoen, 2011; Bolstad *et al.*, 2013). In addition to these, cTnI assays can be affected by some analyte-dependent interferences such as heparin, which due to its negative charge may attract the positively charged cTnI at physiological pH (Gerhardt *et al.*, 2000; Stiegler *et al.*, 2000; Speth *et al.*, 2002); ethylenediaminetetraacetic acid (EDTA), which may dissociate Ca^{2+} from TnC and thus change the conformation of complexed cTnI (Katrukha *et al.*, 1997; Uettwiller-Geiger *et al.*, 2002); and cTnAAbs, which will be discussed in detail in Chapter 2.5. All of these are inherently more critical at low cTnI concentrations detected by the new generation of higher sensitivity assays (Morrow and Antman, 2009; Thygesen *et al.*, 2012b; Korley and Jaffe, 2013; Panteghini, 2013). Thus, it has become necessary to re-evaluate the effects of different preanalytical and analytical factors on cTnI testing (Morrow and Antman, 2009). This was recently highlighted by Pfäfflin (2009) who reported that a novel high-sensitivity cTnI assay has unexpectedly high prevalence of heterophilic antibody interference (3%) compared to the 0.05% prevalence reported for conventional assays.

Due to the molecular heterogeneity of the cTnI molecule, its homology with other cTn and skTn isoforms, and its interactions with other Tn subunits and interfering molecules, one of the main challenges in cTnI detection and in cTnI assay standardization is assay antibody selection. The selection is guided by IFCC, which recommends the use of antibodies whose epitopes are located in the stable midfragment of cTnI and are not affected by complex formation and posttranslational modifications (Panteghini *et al.*, 2001). Although most of the assays target midfragment epitopes and measure approximately the same molecular entity, different sets of capture and detection antibodies display variable immunoreactivity and even loss of reactivity against various cTnI forms and/or are differently affected by various preanalytical and analytical factors. Such variability in immunoreactivity leads to altered signal generation with different sandwich-type cTnI immunoassays and, eventually, unequivocal cTnI results (Wu *et al.*, 1998; Katrukha *et al.*, 1999).

Another major issue contributing to the lack of standardized cTnI assays is the use of different calibration materials and methods. Standardization is based on metrological traceability through a reference measurement system (**Figure 5**) (Panteghini, 2009). This chain begins with a primary reference method, which assigns quantity values to a primary reference material. The primary reference material is needed to calibrate a higher-order reference assay, which in turn is used to establish values to matrix-based secondary reference materials. The secondary reference materials are then used by manufacturers to assign values for their own calibrators. In collaboration with the National Institute of Standards and Technology (NIST), the AACC cTnI Standardization Committee began cTnI standardization efforts by examining a number of candidate primary reference materials, from which a native cardiac troponin complex (ITC) purified from human heart was chosen (Bunk *et al.*, 2000; Christenson *et al.*, 2001; Bunk and Welch, 2006). This cTnI material was designated as NIST standard reference material (SRM) 2921. Highly purified SRM 2921, however, does not seem to be fully representative of the endogenous cTnI found in patients' circulation, and consequently, the use of SRM 2921 as a common calibrator in

commercial systems has not improved the comparability of cTnI measurement results (Christenson *et al.*, 2006). One example of the differences between SRM 2921 and endogenous cTnI is the better stability of endogenous cTnI in the plasma of ACS patients than that of spiked SRM 2921 suggesting that the modification processes of cTnI might already be completed for native cTnI while not for SRM 2921 (Cobbaert *et al.*, 2008). Nevertheless, the non-commutability of SRM 2921 is acceptable when considering its intended use as a primary reference material, i.e. it will not be used directly for assigning values for the working calibrators of field assays (Christenson *et al.*, 2012). Instead, this is planned to be accomplished by a panel of three cTnI-positive serum pools (Tate *et al.*, 2002; Tate *et al.*, 2010). Additionally, the development of a non-commercial enzyme-linked immunosorbent assay (ELISA) to be used as the higher-order reference procedure with specificity for the midfragment epitopes of cTnI, aa 41-49 and 83-93 was recently initiated (Noble *et al.*, 2010) as well as a Western Blot method to characterize the secondary reference samples (He *et al.*, 2011). Despite the continuous efforts on cTnI standardization for more than a decade, the process of conforming uniformity and easy comparison between measurement methods remains an unmet target. It has also been suspected that it will never be achieved unless common antibodies are selected for all cTnI assays (Panteghini, 2005; Jaffe *et al.*, 2006; Apple, 2012; Salvagno *et al.*, 2014). Moreover, the poor correlation observed among the large majority of cTnI assays does not allow for correction factors that would adequately harmonize cTnI results (Apple *et al.*, 2012b).

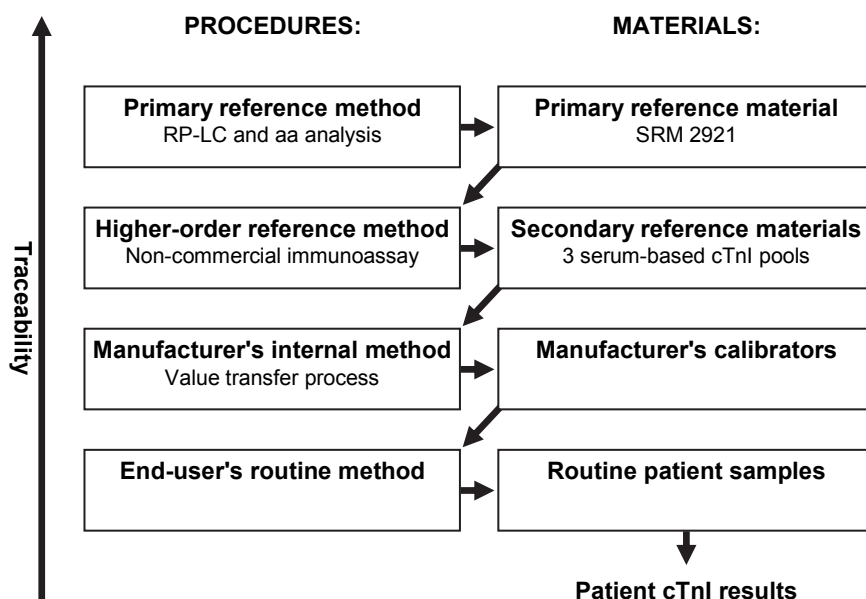


Figure 5. Reference measurement system suggested for cTnI assays (adapted from Panteghini *et al.*, 2008; Panteghini, 2009). RP-LC, reversed-phase liquid chromatography.

Until recently, cTnI assays were not sufficiently sensitive and precise to determine the 99th percentiles of healthy individuals. Therefore, the use of lowest concentrations measurable with a 10% CV was adopted as an alternative approach (Apple *et al.*, 2002). Hence, an additional problem related to the use of cTnI assays is that although the current generation assays can reliably determine the 99th percentile cutoff values and the use of the 99th percentiles in clinical practice has been shown to optimize the sensitivity and specificity of the assays (Keller *et al.*, 2009; Keller *et al.*, 2011; Mills *et al.*, 2011), a range of differently derived cutoff values are still randomly employed resulting in inconsistency in MI diagnosis. According to the second Cardiac Marker Guideline Uptake in Europe (CARMAGUE) study performed after the publication of the second Universal Definition of MI in 2007, the majority of laboratories used either the 10% CV (41.1%) or the 99th percentile (37.9%) as the decision limit (Collinson *et al.*, 2012b). Moreover, the 99th percentile for the cTnI assay used should ideally be established in each laboratory by an appropriately powered study. However, as most laboratories do not have the required resources, the 99th percentile values are generally adopted from the manufacturers' package inserts and peer-reviewed publications. Unfortunately, there is very little consistency in reference sample selection, which may have a dramatic impact on the 99th percentiles obtained (Apple *et al.*, 2012b; Koerbin *et al.*, 2013). Thus far, the majority of reference interval studies have relied on community-based general population cohorts of putatively healthy individuals identified by screening checklists without performing a physical examination, ECG or laboratory testing. Because it has been recently demonstrated that a more stringent selection of reference population decreases the derived 99th percentile with higher sensitivity assays (Collinson *et al.*, 2012a; Koerbin *et al.*, 2013), universal guidelines on how to establish the correct 99th percentile according to age, sex, ethnicity and the number of study participants required in each category are clearly needed. Although using gender-specific and age-adjusted cutoff values, for example, is still under debate, new criteria was recently introduced (Apple *et al.*, 2012a; Sandoval and Apple, 2013) proposing that a minimum of 300 individuals per group are needed to appropriately determine the cutoff and that more thorough screening (e.g. with ECG) is necessary to detect any underlying cardiac conditions. Implementing more stringent selection rules will, however, greatly increase the cost of performing a normal range study. Therefore, providing samples from well characterized healthy subjects for all assay manufacturers to establish their 99th percentile against a common reference population with fixed size and clinical characteristics has been proposed (Newby *et al.*, 2012; Korley and Jaffe, 2013; Wu and Christenson, 2013).

To summarize the goals of cTnI assay development discussed thus far, the ideal characteristics of cTnI assays suitable for routine clinical practice are listed in **Table 5**.

Table 5. Ideal characteristics of cTnI assays.

High-sensitivity
No difference in recognizing various cTnI forms found in the circulation
No cross-reaction with structurally related proteins
No susceptibility to preanalytical and analytical factors
Rapid turnaround time (<60 min)
Simple to perform and cost-effective

2.5 Cardiac troponin specific autoantibodies

The presence of autoantibodies is the consequence of breakdown of self-tolerance towards the corresponding autoantigens. Autoantibodies are an important serological feature of autoimmune diseases but they are also found in cancer, during massive tissue damage and even in apparently healthy subjects (Lleo *et al.*, 2010). cTnAAbs were initially discovered due to their negative interference in cTnI assays. Bohner *et al.* (1996) first attributed false-negative cTnI results to the presence of cTnI-specific cTnAAbs in one patient who had undergone elective coronary artery bypass graft (CABG). In this patient, no cTnI was detected after surgery even though his post-operative cTnT and CK-MB increased as expected. Additionally, the authors were unable to recover cTnI spiked into his pre-operative samples unless the samples were depleted of IgG. Therefore, the falsely low cTnI results were suspected to be caused by circulating IgG with high affinity for cTnI. A few years later, Eriksson *et al.* (2003; 2005a) associated decreased cTnI recoveries seen in blood samples of apparently healthy individuals and noncardiac and cardiac patients to an intrinsic factor that was later identified as cTnAAbs. Their results also indicated that these autoantibodies can mask the cTnI release in MI patients, especially when the amount of cTnI present is low (Eriksson *et al.*, 2003; Eriksson *et al.*, 2005b; Eriksson *et al.*, 2005c). Severe underestimations of endogenous cTnI were recently demonstrated using five conventional cTnI assays in MI patients with cTnI-specific cTnAAbs, even to the extent that these patients were falsely designated as cTnI-negative in relation to the recommended cutoffs (Tang *et al.*, 2012). Although cTnI measurements do not independently rule in or rule out MI, cTnAAbs may have a remarkable effect on acute patient care e.g. by delaying the initiation of treatment.

Currently the mechanisms for the appearance and maintenance of cTnAAbs are not known. As cTns are predominantly localized inside cardiomyocytes, it is possible that an autoimmune reaction can be triggered in response to any initial cardiac injury and subsequent exposure to these previously concealed self-antigens following e.g. inflammation, ischemia, extreme physical endurance training, cardiac incision, or a toxic agent. Therefore, the presence of cTnAAbs in apparently healthy individuals as a consequence of silent cTn release is not surprising. However, the recent demonstration of low but measurable cTn concentrations in most individuals seems to be incompatible with this hypothesis and the vast majority of cardiac patients do not

develop cTn-specific autoimmunity regardless of their exposure to the cTn leakage (Pettersson *et al.*, 2009; Dungen *et al.*, 2010; Lindahl *et al.*, 2010). As the autoimmunity seems to appear only in some individuals, it is likely that genetic and non-genetic susceptibility factors are involved in initiating the formation of cTnAAb. In other words, something more than cTn release is needed for a breakdown in self-tolerance. When autoimmune response is initiated, any subsequent cTn leakage may serve as a booster, which can increase cTnAAb titers in blood and improve affinity of formed autoantibodies (Leuschner *et al.*, 2008; Pettersson *et al.*, 2009; Lindahl *et al.*, 2010).

Detailed data on the molecular characteristics of human cTnAAb are scarce. According to Eriksson *et al.* (2003; 2004), cTnI-specific cTnAAb most commonly target the midfragment of the cTnI molecule, i.e. the IFCC-recommended target for antibodies used in cTnI assays, and especially to the C-terminal region of the midfragment. Furthermore, the authors were able to partly circumvent cTnAAb interference by designing a novel 2+1-type cTnI assay using two capture antibodies against the midfragment and C-terminus of the cTnI molecule (epitopes at aa 41-49 and 190-196, respectively), and one detector antibody against the C-terminus (epitope at aa 137-148, according to the manufacturer, but was recently corrected to 169-178) (Eriksson *et al.*, 2005c; Vylegzhanina *et al.*, 2013). The assay failed to eliminate cTnAAb interference completely, and it is, therefore, likely that the interference is even more heterogeneous than the authors first suspected. In fact, a more recent epitope screening of cTnI-specific autoantibodies showed that the interference encompasses the entire cTnI molecule (Adamczyk *et al.*, 2009a), and contrary to the first results of Eriksson and her co-workers, the strongest cTnAAb responses were now seen against the epitopes at the N- and C-terminal regions of cTnI.

In addition to being able to interfere with cTnI detection by immunoassays designed according to the IFCC-recommended midfragment approach, the presence of cTnAAb may also have either a stabilizing effect on the half-life of circulating cTnI leading to detectable persistent elevations not correlating with the patients' clinical status, which can be seen as false-positive cTnI results, or may directly induce chronic myocardial damage leading to continuous cTnI leakage (Plebani *et al.*, 2002; Pettersson *et al.*, 2009; Lindahl *et al.*, 2010; Michielsen *et al.*, 2011; Wong *et al.*, 2014). Although less explored, the link between cTnI elevations and cTnAAb-positivity is intriguing as persistent low cTnI levels have been shown to predict long-term cardiac mortality (Eggers *et al.*, 2007). The possible clinical significance of cTnAAb is discussed in greater detail in Chapter 2.5.2.

2.5.1 Detection of cardiac troponin specific autoantibodies

Although the exact etiology of cTnAAb is not understood, their occurrence in cardiac patients as well as in apparently healthy individuals has been demonstrated by various methods as summarized in **Table 6**. Despite the fact that the majority of the published methods are immunoassays for detecting IgG class autoantibodies, they vary

substantially in specificity and sensitivity due to the use of different antigens and assay formats. Directly coated cTnI or cTnT surfaces, which is the common approach used in traditional serology assays, are utilized in many of the assays while others use antigen-binding capture surfaces and limit the error due to nonspecific binding of other human antibodies by correcting the results with sample-specific backgrounds obtained without antigen addition. Unlike the other assays, the cTnAAb assay employed also in this thesis (Eriksson *et al.*, 2005a; Pettersson *et al.*, 2009) uses ITC as a target molecule and therefore, cannot differentiate between cTnI-specific and cTnT-specific autoantibodies. Furthermore, the assay sensitivities cannot be determined because there are no defined standards, and various criteria have been used for defining cTnAAb-positivity. Even though the cTnAAb prevalences listed cannot be directly compared between different reports, cTnAAbs can be found in a high proportion (up to 20%) of individuals with or without cardiac diseases.

Table 6. Prevalence of cTnAAb in different study cohorts.

Measurement method (reference)	Antigen	cTnAAb class	Study population	cTnAAb prevalence	Criteria for cTnAAb-positivity
Recovery with cTnI assay (Eriksson <i>et al.</i> , 2003)	N/A	N/A	575 noncardiac patients	4%	Recovery <10%
			475 patients with ACS symptoms	3%	
ELISA (Shmilovich <i>et al.</i> , 2007)	cTnI	IgG	42 apparently healthy individuals	0%	OD >0.43 i.e. mean of healthy individuals +3SD
			32 DCM patients	16%	
			33 ICM patients	18%	
SPR measurement (Landsberger <i>et al.</i> , 2008)	cTnI	IgG	98 apparently healthy individuals	4%	RU >3.7 i.e. mean of healthy individuals +2SD
			98 DCM patients	20%	
			49 ICM patients	18%	
ELISA (Leuschner <i>et al.</i> , 2008)	cTnI	IgG	10 apparently healthy athletes (after ultra-marathon)	0%	Background corrected OD >0 with a sample titer $\geq 1:160$
			272 DCM patients	7%	
			185 ICM patients	9%	
			108 MI patients	9%	
	cTnI	IgM	108 MI patients	1%	
			10 apparently healthy athletes (after ultra-marathon)	0%	
	cTnT	IgG	272 DCM patients	2%	
			185 ICM patients	1%	
			108 MI patients	1%	
			108 MI patients	0%	
	cTnI, cTnT	IgG	95 DCM patients	16%	
			27 DCM patients with advanced heart failure	22%	
TR-FIA (Miettinen <i>et al.</i> , 2008)	cTnI, cTnT	IgG	95 DCM patients	16%	Background corrected fluorescence signal >0 when the T-test gives a P value <0.05
ELISA (Doesch <i>et al.</i> , 2009)	cTnI	IgG	27 DCM patients with advanced heart failure	22%	OD >0
TR-FIA (Pettersson <i>et al.</i> , 2009)	cTnI, cTnT	IgG	81 STE-ACS patients	11%	Background corrected fluorescence signal >100 when the T-test gives a P value <0.05

Measurement method (reference)	Antigen	cTnAAb class	Study population	cTnAAb prevalence	Criteria for cTnAAb-positivity
Chemiluminescent assay (Adamezyk <i>et al.</i> , 2009a)	cTnI	IgG	750 apparently healthy individuals	13%	
			173 cTnI-positive patients	10%	
			200 BNP-positive patients	11%	
			264 Chagas' disease patients	12%	S/LC >6.7 i.e. upper quartile of healthy individuals +1.5IQR
			200 HCV-positive patients	14%	
			50 HBV-positive patients	14%	
			137 RA patients	20%	
			136 SLE patients	10%	
Chemiluminescent assay (Adamezyk <i>et al.</i> , 2009b)	cTnT	IgG	467 apparently healthy individuals	10%	S/LC >5.3 i.e. upper quartile of healthy individuals +1.5IQR
Chemiluminescent assay (Adamezyk <i>et al.</i> , 2010)	cTnI	IgG	345 apparently healthy individuals	10%	
	cTnT	IgG	345 apparently healthy individuals	8%	See above
	cTnI and/or cTnT	IgG	345 apparently healthy individuals	16%	
Chemiluminescent assay (Düngen <i>et al.</i> , 2010)	cTnI	IgG	300 apparently healthy individuals	9%	RVU >1.7 i.e. upper quartile of healthy individuals +1.5IQR
			138 heart failure patients	9%	
TR-FIA (Lindahl <i>et al.</i> , 2010)	cTnI, cTnT	IgG	957 STE-ACS patients	7%	Background corrected fluorescence signal >100 when the T-test gives a P value <0.05
ELISA (Doesch <i>et al.</i> , 2011)	cTnI	IgG	249 DCM patients	17%	Background corrected OD >0 with a sample titer $\geq 1:80$
			141 ICM patients	21%	
ELISA (Lappé <i>et al.</i> , 2011)	cTnI	IgG	35 apparently healthy individuals	20%	OD ≥ 2.5 times the average background at a sample titer of 1:1000 & the signal increase and decrease with increasing and decreasing sample concentrations, respectively
			44 DCM patients	14%	
ELISA (Tang <i>et al.</i> , 2012)	cTnI	IgG	210 apparently healthy individuals	0%	OD >0.55 i.e. mean of healthy individuals +3SD
			121 MI patients	15%	

Measurement method (reference)	Antigen	cTnAAb class	Study population	cTnAAb prevalence	Criteria for cTnAAb-positivity
Western blot (Tang <i>et al.</i> , 2012)	cTnI	IgG	121 MI patients	11%	Detectable cTnI-specific band(s)
TR-FIA (Eerola <i>et al.</i> , 2013)	cTnI, cTnT	IgG	74 apparently healthy children or adolescents	0%	Background corrected fluorescence signal >100 when the T-test gives a P value <0.05
			138 children or adolescents with congenital heart defect	1%	
TR-FIA (Eerola <i>et al.</i> , 2014)	cTnI, cTnT	IgG	34 apparently healthy children	0%	Background corrected fluorescence signal >100 when the T-test gives a P value <0.05
			38 children with hypoplastic left heart syndrome	0%	

N/A, not applicable; OD, optical density; SD, standard deviation; DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; SPR, surface plasmon resonance; RU, resonance units; TR-FIA, time-resolved fluoroimmunoassay; S/LC, signal-to-low control response; IQR, interquartile range; BNP, brain natriuretic peptide; HCV, hepatitis C virus; HBV, hepatitis B virus; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; RVU, relative value units

2.5.2 Pathophysiological and clinical relevance

The existence of circulating autoantibodies against various heart-specific antigens is well established and accumulating evidence suggest that humoral and/or cellular autoimmune responses play a pivotal role in the etiology of cardiac diseases, particularly in myocarditis, DCM and heart failure (Okazaki and Honjo, 2005; Caforio *et al.*, 2008; Lappé *et al.*, 2008; Wehlou and Delanghe, 2009; Kaya *et al.*, 2010). Because autoantibodies are generally capable of causing direct cellular damage by inducing apoptosis, complement activation and cell-mediated cytotoxicity, heart-reactive antibodies may have an active role in the etiology of cardiac diseases. Alternatively, these autoantibodies may merely reflect the ongoing myocardial damage and protein release with no causal association with cardiac pathology. In both cases, heart-specific autoantibodies including cTnAAbs could serve as biomarkers of the underlying disease processes. Because unraveling the mechanisms and implications of heart-specific autoimmune responses and roles of heart-specific autoantibodies could aid the development of new approaches for therapy and risk stratification of cardiac diseases, they have been increasingly studied.

2.5.2.1 Mouse models

Recent studies on mice have associated cTnI-specific cTnAAbs and T cells with cardiac inflammation and dysfunction suggesting a direct role for cTnI-specific autoimmunity in the pathogenesis of cardiac diseases. In 2001, it was first reported that programmed cell death-1 (PD-1) receptor deficient mice develop severe DCM with production of high-titered autoantibodies against a heart-specific, 30-kDa protein that was subsequently identified as cTnI (Nishimura *et al.*, 2001; Okazaki *et al.*, 2003). Further studies by this group demonstrated that the administration of cTnI-specific Mabs to wild-type mice induced cardiac dilatation and dysfunction (Okazaki *et al.*, 2003). The authors suggested that the observed cardiac phenotype is caused by the chronic stimulation of Ca^{2+} influx in cardiomyocytes, because cTnI, in contrast to cTnT, was not restricted to the cytoplasm but also found on the surface of mouse ventricular myocytes and because cTnI-specific Mabs were found to augment the voltage-dependent L-type Ca^{2+} current.

Soon after, it was reported that the immunization of wild-type mice with cTnI induces a robust autoimmune reaction encompassing both humoral and cellular responses leading to severe myocardial inflammation, fibrosis, cardiac dysfunction and increased mortality (Göser *et al.*, 2006). Although comparable levels of autoimmune responses were induced by cTnT, cTnT-immunized mice did not develop the symptoms of cardiac disease. It was speculated that this is because cTnI-specific cTnAAbs and T cells can bind to the target protein on the surface of cardiomyocytes whereas cTnT-specific cTnAAbs and T cells cannot. Additionally, cTnI-immunized mice later developed high titers of autoantibodies to cardiac myosin which indicates that primary myocardial injury with the release of other cardiac proteins may broaden the immune response and aggravate autoimmune-mediated cardiac damage. Furthermore, the

authors demonstrated that pre-immunization with cTnI before chronic ligation of the left anterior descending coronary artery increased infarct size and post-infarct fibrosis and inflammation. Later, Volz *et al.* (2011) showed in wild-type mice that these deleterious effects endured beyond the early period, as late as 6 months after MI, and also exacerbated ischemia/reperfusion injury. These results indicate that the presence of cTnI-induced autoimmune response might render patients more vulnerable to prospective myocardial injury.

A similar phenotype as seen with cTnI immunization was also obtained by transferring stimulated cTnI-specific T cells to wild-type mice (Kaya *et al.*, 2008). This observation suggests that the initiation of the inflammatory process in myocardium followed by fibrosis and alteration of heart function is primarily T-cell dependent which is supported by the notion that mice administered with cTnI-specific Mabs showed no inflammation (Okazaki *et al.*, 2003). The authors also identified two pathogenic mouse cTnI sequences, aa 105-122 and 131-148, which are responsible for disease induction. On the other hand, one study demonstrated that relatively mild cardiac damage and cTnI release in the acute phase of experimental coxsackievirus B3 induced myocarditis may lead to the development of cTnAAbs (Latva-Hirvelä *et al.*, 2009). However, the presence of cTnAAbs was not associated with more severe myocarditis or decreased contractility of the heart when compared to cTnAAb-negative mice.

2.5.2.2 Humans

The possible clinical consequences of circulating cTnAAbs have been studied also in humans. Two studies on NSTEMI-ACS patients demonstrated that the presence of cTnAAbs may lead to a higher and longer cTnI release (Pettersson *et al.*, 2009; Lindahl *et al.*, 2010) but otherwise the role of cTnAAbs has remained controversial. One study on MI patients indicated that cTnI-specific cTnAAbs may have a negative effect on the recovery of cardiac function; cTnAAb-positive patients did not show any significant improvement in left ventricular function after an ischemic event while cTnAAb-negative patients did (Leuschner *et al.*, 2008). Another study suggested a potential protective effect of cTnAAbs as cTnI-specific cTnAAbs were associated with improved survival of patients with DMC (Doesch *et al.*, 2011). This surprising finding was in accordance with the group's previous study on DCM patients with advanced heart failure, where cTnI-specific cTnAAbs at baseline were associated with a lower therapeutic benefit of immunoadsorption therapy (Doesch *et al.*, 2009). Most of the published studies, however, have not found a significant association between the presence of circulating cTnAAbs and adverse outcome in cardiac patients (Shmilovich *et al.*, 2007; Miettinen *et al.*, 2008; Düngen *et al.*, 2010; Lindahl *et al.*, 2010).

The only study on cTnI-specific cellular-based autoimmune responses indicated that these could contribute to the pathogenesis of DCM (Lappé *et al.*, 2011). The authors demonstrated a greater likelihood of identifying a cellular proliferative response

against cTnI in stable DCM patients, in up to one-fifth of the patients, than in healthy controls. Additionally, they did not identify any significant correlations between the presence of cTnI-specific cTnAAbs and a positive cellular response, which challenges the idea of using cTnAAb measurements to detect the presence of potentially pathogenic autoimmunity.

The low number of cTnAAb-positive patients and adverse end points in these publications prevent us from making any definite conclusions on the impact of circulating cTnAAbs. Furthermore, autoantibodies present only one part of the immune response, and further studies on cellular responses are clearly required. Therefore, the role of humoral and cellular cTn-responses remains to be elucidated through larger clinical trials and adequate follow-up times. Without combined information, it is difficult to assess whether cTnAAbs represent a primary cause, a secondary response to ongoing myocardial injury caused by other processes, or predisposition to the development of cardiac disease. The discrepancy between the reports may also be related to the nature of the epitopes targeted by cTnAAbs; the results do not differentiate between cTnAAb subsets and potentially there are only some cTnI sequences which have clinical effects while others do not, as has been previously shown in mice (Kaya *et al.*, 2008).

3 AIMS OF THE STUDY

The primary aim of this thesis was to develop and optimize immunoassays for cTnI and cTnAAb determination which could then be used in the future to explore the clinical impact of circulating cTnAAbs on cTnI testing and to unravel the etiology of cTn-related autoimmune responses. A secondary objective was to obtain detailed data on the prevalence and molecular characteristics of cTnAAbs.

More specifically, the aims in the original publications were:

- I** To study the extent of cTnAAb interference on different cTnI assay configurations and to identify antibody combinations minimally affected by cTnAAbs.
- II** To investigate the molecular characteristics of human cTnAAbs.
- III** To validate an optimized cTnAAb assay and to determine the prevalence of cTnAAbs in consecutive chest pain patients presenting to an emergency department, i.e. in the population where cTnI assays are typically applied
- IV** To validate a sensitive cTnI assay free from cTnAAb interference.

4 SUMMARY OF MATERIALS AND METHODS

A summary with some additional information is presented here while the details of the materials and methods used in this study are described in the original publications **I-IV**.

4.1 Samples

The various sample panels used in publications **I-IV** are described in **Table 7**. Additional heparin plasma and serum samples were obtained from chest pain patients admitted to Central Ostrobothnia Central Hospital (Kokkola, Finland) and from apparently healthy volunteers at the Department of Biotechnology, University of Turku (DBUT) (Turku, Finland) in 2010-2013. All samples were collected according to normal laboratory routines with informed consent of the participant. Clinical patients were treated according to routine hospital protocols, and the retrospective evaluation and clinical endpoints used in publication **III** are illustrated in the original paper. The study protocols were conducted in accordance with the Declaration of Helsinki as revised in 1996 and approved by the local ethics committees.

Table 7. Sample panels used in the original publications **I-IV**.

Collection place	Collection time	Study population	Sample matrix	Publication
S:t Görans Hospital, DBUT	2007-2008	1,105 noncardiac patients and apparently healthy individuals	Heparin plasma, serum	I
58 Scandinavian hospitals (FRISC-II trial)	1996-1998	210 NSTEMI-ACS patients (admission)	EDTA plasma	I
DBUT	2010-2011	20 apparently healthy individuals	Serum	II
9 Finnish hospitals	1998-2000	28 NSTEMI-ACS patients (admission and 3-month follow-up)	Serum	II
Turku University Hospital	2000-2001	510 patients with ACS symptoms (admission)	EDTA plasma	III
European and North American hospitals (GUSTO-IV trial)	1999-2000	250 NSTEMI-ACS patients	Serum	IV
Labquality (NORIP study)		159 apparently healthy individuals	Serum	IV

FRISC, Fragmin and Fast Revascularization during Instability in Coronary artery disease; GUSTO, Global Utilization of Strategies to Open Occluded Arteries; NORIP, Nordic Reference Interval Project

4.2 Antibodies

4.2.1 Monoclonal antibodies and antibody fragments

All Mabs except 8I7 (International Point of Care, Toronto, Canada) were kindly provided by HyTest Ltd (Turku, Finland). Recombinant antigen-binding fragments (Fab) 4C2 and MF4 of correspondingly named Mabs were cloned from the hybridoma cell lines of HyTest Ltd, and Fab 9707 from the hybridoma cell line of Medix Biochemica (Kauniainen, Finland). A summary of antibodies used in publications **I-IV**, their antigens and their epitopes are presented in **Table 8**.

Table 8. Antibodies and their binding sites used in the original publications **I-IV**.

Code	Form	Antigen	Binding site	Publication(s)
P4-14G6	Mab	cTnI	aa 1-15	II
23C6	Mab	cTnI	aa 15-25	II
M18	Mab	cTnI	aa 18-28	I
4C2	Mab/Fab	cTnI	aa 23-29	I, II, III, IV
228	Mab	cTnI	aa 26-35	I, IV
M155	Mab	cTnI	aa 26-35	II, III, IV
10F4	Mab	cTnI	aa 34-37	II
19C7	Mab	cTnI	aa 41-49	I, II, IV
247	Mab	cTnI	aa 65-74	II
560	Mab	cTnI	aa 83-93	I, II, IV
8E10	Mab	cTnI	aa 86-90	I, II
415	Mab	cTnI	aa 104-119	II
84	Mab	cTnI	aa 117-126	II
M46	Mab	cTnI	aa 130-145	II
581	Mab	cTnI	aa 143-152	II
441	Mab	cTnI	aa 148-158	II
8I7	Mab	cTnI	aa 169-178	I, II, III, IV
625	Mab	cTnI	aa 169-178	II
267	Mab	cTnI	aa 169-178	I
472	Mab	cTnI	aa 182-191	II
9707	Fab	cTnI	aa 190-196	I
MF4	Mab/Fab	cTnI	aa 190-196	I, II, III, IV
P45-10	Mab	cTnI	aa 195-209	II
7B9	Mab	TnC	Unknown	II
3D3	Mab	Human IgG	Constant region	I, II, III, IV
2C11	Mab	Human IgG1	Constant region	II
3C7	Mab	Human IgG2	Hinge region	II
5G12	Mab	Human IgG3	Hinge region	II
5C7	Mab	Human IgG4	Constant region	II

A corrected Mab 8I7 epitope was obtained from a recent publication (Vylegzhanina *et al.*, 2013) while other epitope specificities were obtained from the manufacturers' package inserts (the 8I7 epitope of 137-148 reported by the manufacturer was used in the original publications **I-III**). The manufacturers declare no cross-reaction with skTnIs for other cTnI-specific antibodies used except for Mabs M46 (<10%), 267 (<10%) and 472 (<50%). In 2013, however, Mab 8I7 was reported to recognize the mixture of slow- and fast-twitch skTnI (data was not shown) (Vylegzhanina *et al.*, 2013).

4.2.2 Labeling with lanthanide chelate and biotin

Tracer Mabs were labeled with a 25- to 75-fold molar excess of intrinsically fluorescent 9-dentate α -galactose europium chelate (von Lode *et al.*, 2003) (Radiometer/Innotrac Diagnostics Oy, Turku, Finland or DBUT) (**I-IV**). Capture Mabs were biotinylated with a 10- to 30-fold molar excess of biotin isothiocyanate (DBUT) (**I-IV**) or with a 200-fold molar excess of biotin-caproylhydrazine (Sigma, St. Louis, MO, US) (**I**). The labeled Mabs were separated from free chelate or biotin by gel filtration using Tris-buffered saline with azide (TSA) (50 mmol/L Tris-HCl, pH 7.75, 150 mmol/L NaCl and 0.5 g/L NaN₃) for elution. Capture Fabs were site-specifically biotinylated with 95 μ mol/L maleimide-PEO₂-biotin (Thermo Fisher Scientific, Waltham, MA, US) (**I** and **IV**) during protein purification and finally transferred to TSA. All europium labeled or biotinylated antibodies were stabilized with bovine serum albumin (BSA) (1 g/L).

4.3 Other reagents

Human ITC from HyTest Ltd was used for spiking in analytical recovery experiments (**I**, **II** and **IV**), for cTnI assay calibration (**I** and **IV**), and as a target molecule in cTnAAb assays (**I-IV**). Standards were prepared by diluting ITC into TSA containing 75 g/L BSA. The cTnI concentration of the ITC stock reported by the manufacturer was used to assign the cTnI concentrations of the dilutions.

Normal capacity streptavidin (SA) plates were purchased from Kaivogen Oy (Turku, Finland) (**I-IV**). High capacity sulphydryl(SH)-SA plates were produced as described earlier (Ylikotila *et al.*, 2006) (**I**).

4.4 Antibody-coated spots

A schematic representation of antibody-coated spots used in publication **IV** is shown in **Figure 6**. Biotinylated capture antibodies, 33 mg/L of each Fab 4C2, Mab 19C7 and Fab MF4 in TSA containing 10 g/L glycerol, were printed (150 droplets of ~250 pL/spot, room temperature, 70% humidity) onto the bottom of SA-coated microtiter wells using Nano-Plotter NP 2.1 (GeSiM, Grosserkmannsdorf, Germany). After an 1-h incubation in a closed humidity chamber and washings, 40 μ L of Insulating Layer II buffer solution (ILII) (Innotrac Diagnostics Oy) supplemented with

62.5 mmol/L Tris (pH 8.5) were added into the wells and the wells were dried overnight at +35°C.

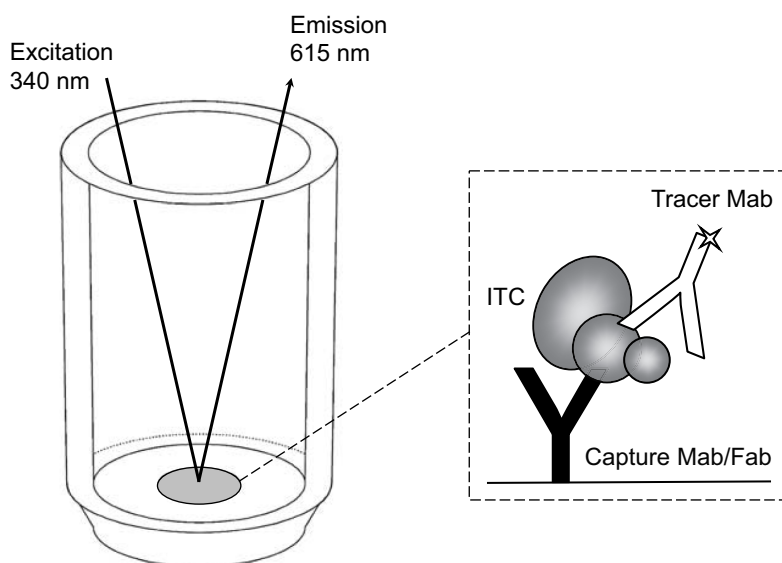


Figure 6. Schematic presentation of the antibody-coated spots used in the original publication **IV**. A spot-type binding surface concentrates the sandwich formation to coincide more closely with the excitation beam of the fluorometer, which results in a 5- to 7-fold increase in assay sensitivity compared to the commonly used whole well approach (Ylikotila *et al.*, 2005; Ylikotila *et al.*, 2006; Välimaa *et al.*, 2008).

4.5 Immunoassays

4.5.1 Cardiac troponin I

Epitope maps of the investigational and commercial cTnI assays used in publications **I** and **IV** is shown in **Figure 7**.

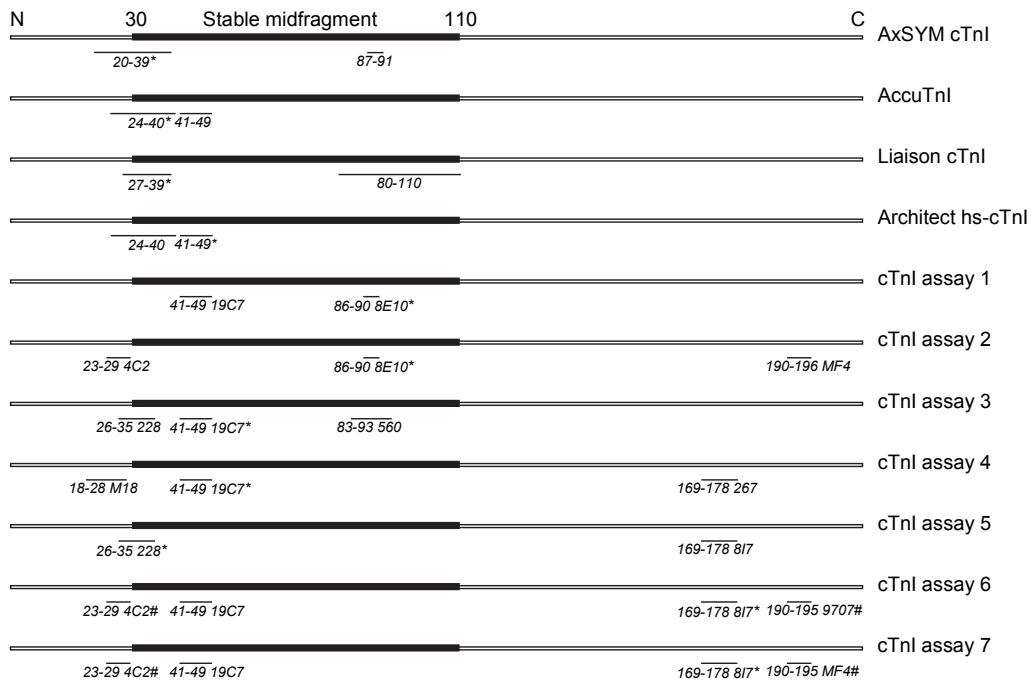


Figure 7. Epitope map of antibodies used in the four commercial and the seven investigational cTnI assays used in the original publications **I** and **IV** (adapted from **I**). The bar next to the assay name represents the linear amino acid sequence of cTnI. The antibody epitopes are marked with shorter lines below the cTnI sequence with respective amino acid locations and antibody codes (*, tracer; #, Fab).

4.5.1.1 Commercial assays

Three conventional midfragment targeting commercial assays, AxSYM first-generation cTnI assay (Abbott Laboratories, Abbott Park, IL, US), AccuTnI second-generation assay (Beckman Coulter, Brea, CA, US) and Liaison cTnI assay (Byk-Sangtec Diagnostica, Dietzenbach, Germany), were used in publication **I**. According to the manufacturers, the analytical sensitivities were 300, 10, and 5 ng/L for AxSYM cTnI, AccuTnI, and Liaison cTnI, respectively. One high-sensitivity midfragment targeting assay, Architect hs-cTnI assay (Abbott Laboratories), was used in publication **IV**. The LoB and LoD of Architect hs-cTnI assay were 0.5 and 1.0-1.2 ng/L, respectively, and the 99th percentile among healthy individuals was 14-23 ng/L (Apple *et al.*, 2012b; Koerbin *et al.*, 2012).

4.5.1.2 Investigational assays

In the regular whole well approach used in cTnI assays 1-6, biotinylated capture antibodies (12.5-200 ng) were first added to SA- or SH-SA-coated microtiter wells in 25 µL of Kaivogen buffer solution (KBS) (Kaivogen Oy) and incubated for 1 h at

room temperature. After washing, 20 μ L of standard or sample and 100 ng of the europium labeled tracer in 20 μ L of ILII were added into the wells. The wells were then incubated for 30 min at +36°C, 900 rpm in EMS incubator/shaker (Thermo Electron Corporation/Labsystems, Helsinki, Finland). Finally, the washed wells were dried and fluorescence was measured in time-resolved mode directly from the surface with Victor X4 Multilabel Counter (Perkin-Elmer/Wallac, Turku, Finland). In the spot-type approach used in cTnI assay 7, 20 μ L of standard or sample and 75 ng of the europium labeled Mab 8I7 in 10 μ L of Innotracs Aio! buffer solution (Innotrac Diagnostics Oy) were added into the antibody-coated spot wells. The wells were then incubated for 3 h at +36°C, 1800 rpm before the washing and fluorescent measurement.

Analytical recoveries were determined by measuring cTnI from the unspiked and ITC-spiked (cTnI assays 1-6: 30,000 ng/L cTnI; cTnI assay 7: 10-50,000 ng/L cTnI) aliquot of each sample after incubating them for 1 h at +4°C. With cTnI assays 1-6, ITC-specific fluorescence signals were directly compared to the fluorescence signal of similarly spiked TSA-BSA (**I**). With cTnI assay 7, analytical recoveries were calculated by comparing the ITC-specific cTnI concentration obtained to the known amount of spiked cTnI (**IV**).

Analytical performance was determined only for cTnI assays 1, 3, 6 and 7. With cTnI assays 1, 3 and 6, sensitivities were estimated based on the standard curves (nonlinear fitting, concentration corresponding to 3SD of blank) (**I**). With cTnI assay 7, LoB, LoD and total precision were determined according to CLSI Guidelines EP17-A2 and EP-A5, and dilution linearity was assessed by serially diluting samples up to 1/243 with analyte-free (cTnI concentration <LoB) sample pool (**IV**). In addition, the stability of endogenous cTnI was assessed with cTnI assay 7 by measuring analyte concentrations from aliquoted cTnI-containing samples after additional thawing and freezing (-20°C) cycles and after incubating them at +4°C or at room temperature.

4.5.2 Comparing capture efficiencies of cardiac troponin I assays

In publication **I**, the differences observed in the calculated cTnI values of NSTEMI-ACS patients were further investigated by comparing the relative capture efficiencies of cTnI assays 1 and 6 to standards and cTnI-containing samples. First, 20 μ L of standard or sample and 20 μ L of ILII were added on the capture surfaces of assay 1 and assay 6, respectively. After a 30 min incubation period (+36°C, 900 rpm), the solution with unbound analyte was transferred to a second set of assay 6 and assay 1 capture surfaces, respectively, and incubated for another 30 min. Bound cTnI was detected from both surfaces by incubating tracer antibodies in 40 μ L of ILII for 30 min before washing and fluorescence measurement.

4.5.3 Determining the binding sites of cardiac troponin specific autoantibodies

In publication **II**, the binding sites of cTnAAbs on the cTnI molecule were determined by comparing the analytical recoveries in cTnAAb-positive samples to the recoveries in cTnAAb-negative samples. The fluorescence signals were measured from the unspiked and ITC-spiked (30,000 ng/L cTnI) aliquots of each sample with sandwich-type immunoassays using various cTnI-specific Mabs as a capture and the common TnC-specific Mab as a tracer. First, 300 ng of the biotinylated capture antibody in 25 μ L of KBS was immobilized in SA-coated microtiter wells and incubated for approximately 1 h at room temperature. Meanwhile, the sample aliquots were incubated for 1 h at +4°C. After washing the wells, 20 μ L of sample and 100 ng of the europium labeled Mab 7B9 in 20 μ L of ILII were added. The wells were then incubated for 1 h at +36°C, 900 rpm before washing and fluorescence measurement. Finally, sample specific recoveries for different capture epitopes were calculated by comparing the ITC-specific fluorescence signal of each sample to the mean signal of cTnAAb-negative samples.

4.5.4 Cardiac troponin specific autoantibodies

A schematic representation of the cTnAAb assay design and a summary of different assay versions used in publications **I-IV** are represented in **Figure 8** and **Table 9**, respectively. Samples were first diluted 5-fold with ILII and the fluorescence signal was measured from each prediluted sample with and without ITC-addition (30,000 ng/L cTnI). After binding the possible cTnAAbs to an added ITC complex (1 h, +4°C), 30 μ L of sample and 200 μ L of assay buffer were added into triplicate SA wells preimmobilized with biotinylated capture antibodies. The wells were incubated for 1 h at +36°C, 900 rpm and washed. Subsequently, europium labeled tracer antibody was added into the wells in 200 μ L of assay buffer and the wells were incubated for another 1 h at +36 °C, 900 rpm. Finally, fluorescence was measured in time-resolved mode from the surface of washed and dried wells. Autoantibody positivity was defined as ≥ 0 (**I**) or ≥ 100 (**II**, **III** and **IV**) counts above background (no ITC added) when the T-test gave a P value < 0.05 .

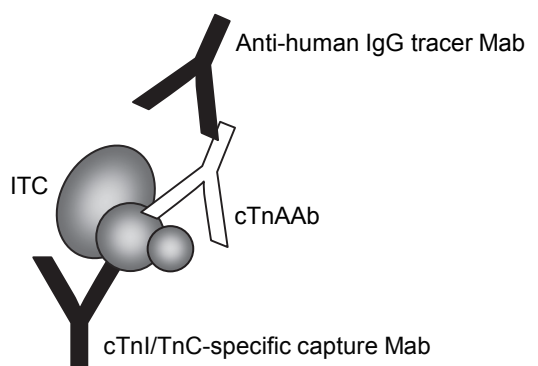


Figure 8. Schematic representation of the cTnAb assay design used in the original publications **I-IV** (adapted from **III**). cTNAbs were first bound to an added ITC complex. The formed ITC-cTnAb complexes were subsequently captured on microtiter well with cTnI- and/or TnC-specific antibodies. Finally, the bound cTNAbs were detected with labeled anti-human IgG tracer.

Table 9. The cTnAb assay versions used in the original publications **I-IV**.

Publication(s)	Name	Capture antibodies	Tracer antibody	Assay buffer
I, II, III	Old*	150 ng Mab 4C2, 150 ng Mab MF4	40 ng Mab 3D3	KBS
II	Total IgG, IgG1, IgG2, IgG3 or IgG4	100 ng Mab 4C2, 100 ng Mab MF4, 100 ng Mab 7B9	50-150 ng Mab 3D3, 2C11, 3C7, 5G12 or 5C7	KBS, 10 mg/L native mouse IgG, 5 mg/L denaturated mouse IgG
III, IV	New	150 ng Mab M155, 150 ng Mab 8I7	40 ng Mab 3D3	KBS, 27 g/L NaCl

*(Eriksson *et al.*, 2005a; Pettersson *et al.*, 2009)

4.6 Statistical analysis

The analyses were performed with STAT View 5.0 (SAS Institute, Gary, NC, US) (**I**), SAS 9.2 (SAS Institute) (**I**), PASW Statistics 18 (SPSS Inc, Chicago, IL, US) (**II** and **III**), IBM SPSS Statistics 21 (IBM, Armonk, NY, US) (**IV**) or Analyse-it 2.30 for Microsoft Excel (Analyse-it Software Ltd, Leeds, United Kingdom) (**IV**). In all statistical tests, two-sided P values <0.05 were considered statistically significant. The statistical analysis methods and tests are specified in the original publications **I-IV**.

5 SUMMARY OF RESULTS AND DISCUSSION

5.1 Troponin-specific autoantibody interference in different cardiac troponin I assay configurations

Presently, most cTnI-assay manufacturers follow the IFCC recommendation and use antibodies binding to the midfragment of the cTnI molecule (Jaffe, 2011; Apple *et al.*, 2012b). Because the midfragment is also most frequently targeted by cTnAAb (Eriksson *et al.*, 2004; Eriksson *et al.*, 2005c), we hypothesized that such cTnI assays are likely to suffer from cTnAAb interference and that this interference can be counteracted by selecting the assay antibodies differently. In publication I, cTnI assays 1-6 using different epitopes across the cTnI molecule were constructed to study the extent of cTnAAb interference in different assay configurations and to identify antibody combinations that would be minimally affected by cTnAAb. The analytical recoveries of 1,105 hospitalized noncardiac patients and apparently healthy individuals were first determined with the midfragment targeting cTnI assay 1. Based on these results, two study groups with lowered recoveries were identified: low recovery (LR) group (<10%, n=49) and medium recovery (MR) group (10%-20%, n=17). A normal recovery (NR) group (n=66) was randomly chosen from the remaining samples. Subsequently, the samples were analyzed with the old cTnAAb assay and cTnI assays 2-6.

All LR, all MR and 24 NR samples were cTnAAb-positive with the cTnAAb signals of each study group differing significantly from each other ($P<0.001$) (**Figure 9A**). Median (25th-75th percentiles) signals in the LR and MR groups were 13,301 (7,444-24,845) and 2,690 (1,297-3,583) counts, respectively, whereas half of the cTnAAb-positive samples in the NR group had cTnAAb signals ≤ 100 counts. The reverse correlation between the study groups demonstrated that the low recoveries obtained with cTnI assay 1 resulted from cTnAAb interference. However, when the sample recoveries of cTnI assays 1-6 were directly calculated from the signals of similarly spiked TSA-BSA, the median recoveries of the NR group (44%-125%) differed notably between the assays studied. This is typical for matrix effects stemming from differences in immunoreactivity of the assay antibodies in the buffer-based standards and test samples, whereas the variation around the medians conceivably characterizes sample-specific interferences shortly discussed in Chapter 2.4.2. To correct the results for the matrix effects, they were normalized with the medians of the NR group set to 100% (the original recovery divided by the median recovery of the NR group and multiplied by 100%). After the normalization, the negative interference of LR and MR samples clearly decreased when alternative epitope combinations deviating from the IFCC-recommended midfragment approach were used (**Figure 9B**). Compared to the median of the NR group (100%), the medians of cTnI assays 1-6 were 9%, 26%, 32%, 53%, 58%, and 103% in the LR group, and 35%, 76%, 78%, 83%, 65%, and 102% in the MR group, respectively. Differences between the study groups remained highly significant ($P<0.001$) in cTnI assays 2-5 but not in assay 6 ($P=0.165$).

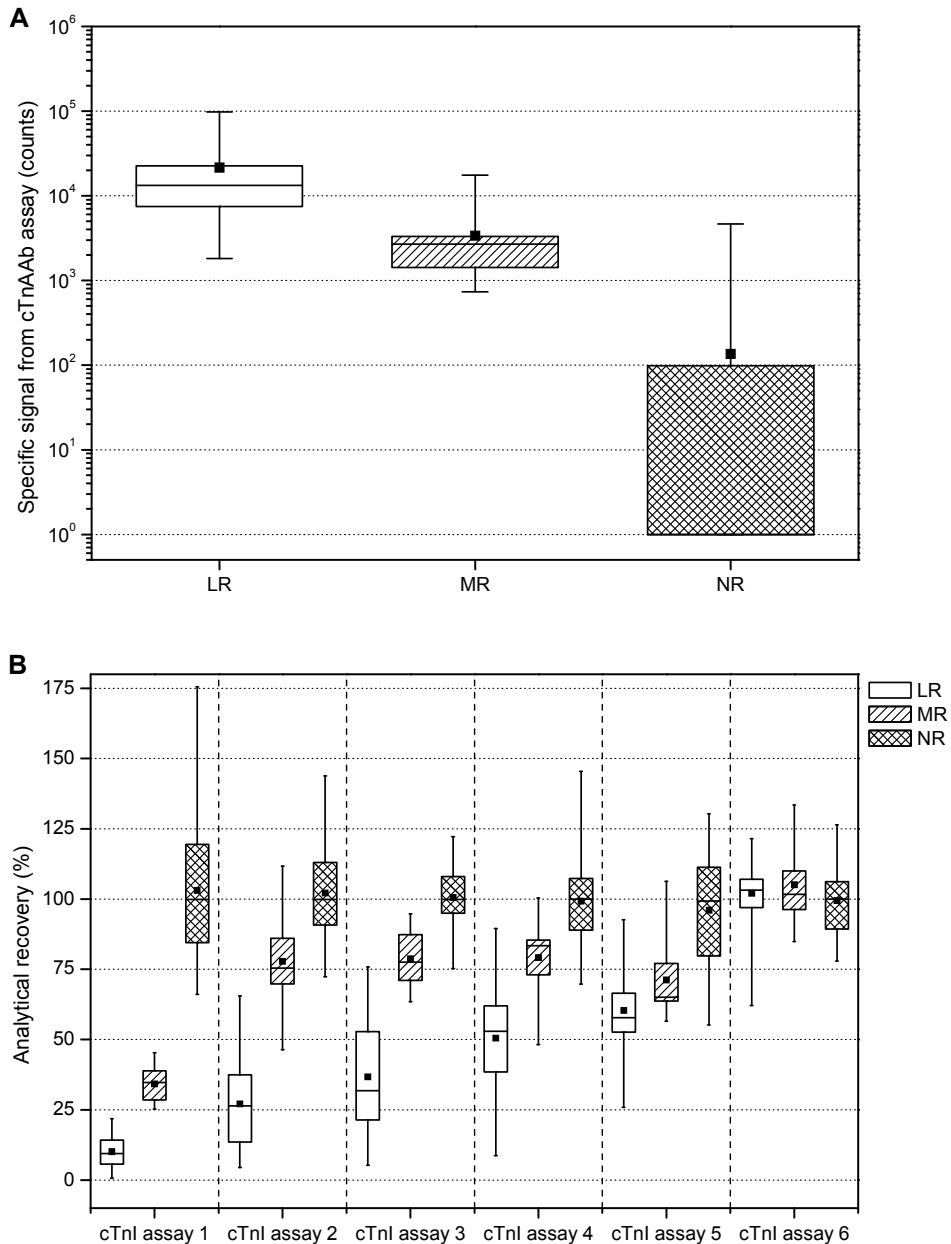


Figure 9. cTnAb signals (A) and normalized analytical recoveries (B) in 132 individuals without known cardiac conditions divided into three study groups according to the recoveries obtained with midfragment targeting cTnI assay 1 (adapted from I). All cTnAb-negative samples have been given a cTnAb signal of 1. Whiskers represent minimum and maximum signals; boxes represent 25th percentile, median, and 75th percentile; and black squares represent means.

The analytical recovery tests showed that the epitope selection had a profound effect on the degree of negative cTnAAb interference which confirmed and elaborated the conclusions of previous reports (Eriksson *et al.*, 2004; Eriksson *et al.*, 2005c). However, highly purified ITC used in the recovery studies is not truly representative of the endogenous cTnI found in the circulation (Christenson *et al.*, 2006; Cobbaert *et al.*, 2008). Therefore, three of the investigational assays were chosen for more detailed characterization with clinical samples obtained from 210 (140 cTnAAb-negative, 70 cTnAAb-positive) NSTEMI-ACS patients. cTnI assays 1 and 3 were selected to represent antibody configurations of current state-of-the-art assays, whereas cTnI assay 6 was selected because of its apparent lack of cTnAAb interference. Their performance was compared to the performance of three conventional commercial kits.

The analytical sensitivities of cTnI assays 1, 3 and 6 were 300, 60 and 20 ng/L, respectively. Because patients with a cTnI concentration lower than the analytical sensitivity of the assay in question were excluded, the final analyses were completed with 108 cTnAAb-negative and 53 cTnAAb-positive samples. **Figures 10A and 10B** show the scatter plots of the cTnI concentrations of these patients measured with cTnI assays 1 and 6 with assay 3 as a reference. cTnI assays 1 and 3 performed similarly, whereas with assay 6 the majority of cTnAAb-positive samples stood out from the scatter because of the higher cTnI values detected with cTnI assay 6. The mean (SD) concentrations of cTnI assays 1, 3 and 6 were 2,770 (3,050), 2,410 (3,140) and 1,130 (1,730) ng/L in the cTnAAb-negative cohort, and 2,210 (2,640), 2,490 (3,270) and 4,150 (6,400) ng/L in the cTnAAb-positive cohort, respectively. Similar scatter plots were obtained when the investigational assays were compared to AxSYM cTnI, AccuTnI and Liaison cTnI assays. Comparison to AccuTnI assay is shown here as an example (**Figures 10C and 10D**). It shows that cTnI assay 3 and AccuTnI performed similarly, whereas the scatter profile between assay 6 and AccuTnI was highly similar to the one seen between cTnI assays 6 and 3.

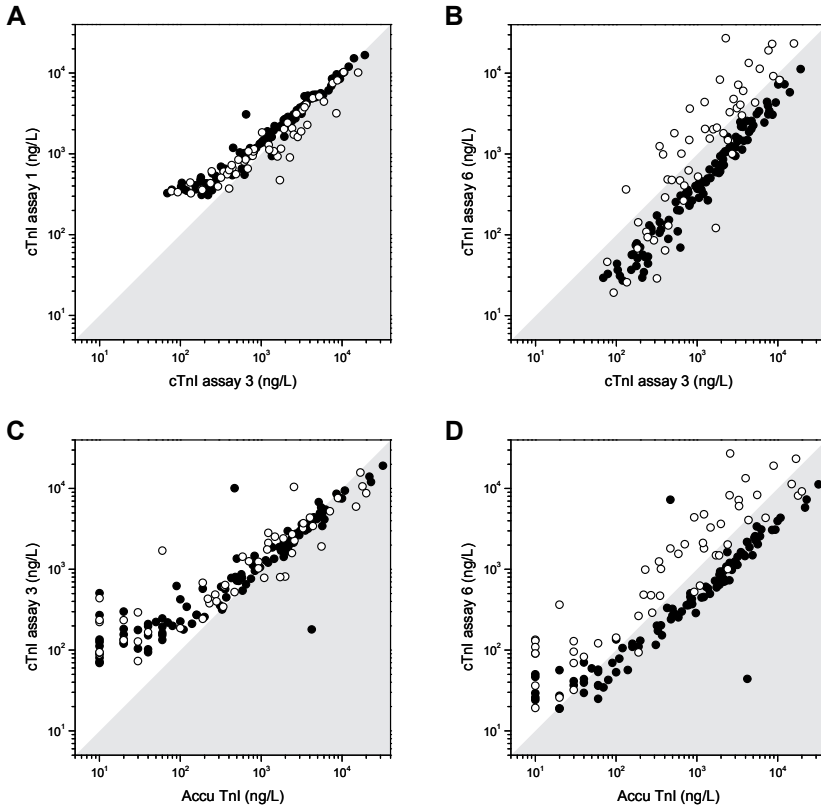


Figure 10. Original cTnI concentrations in 108 cTnAAb-negative (●) and 53 cTnAAb-positive (○) NSTE-ACS patients with cTnI assays 1 (A) and 6 (B) using cTnI assay 3 as a reference, and similarly with cTnI assays 3 (C) and 6 (D) using the commercial AccuTnI assay as a reference (adapted from I).

Although cTnI assays 1, 3 and 6 were calibrated against the same tissue-derived cTnI preparation, the cTnI concentrations of the cTnAAb-negative patients with cTnI assay 3 were on average 0.7-fold and 2.8-fold the concentrations with assays 1 and 6, respectively. Again, to correct the obtained cTnI results for the matrix effects and to enable a comparison of the effects of cTnAAb in the cTnAAb-positive cohort, the data were normalized against the cTnI values obtained with assay 3 for the cTnAAb-negative cohort using the following regression equations: $\ln \text{cTnI assay 1}_{\text{normalized}} = -0.036 \times (\ln \text{cTnI assay 1})^2 + 1.29 \times \ln \text{cTnI assay 1} - 0.51$ and $\text{cTnI assay 6}_{\text{normalized}} = 2.19 \times \text{cTnI assay 6} + 0.11$. When the original cTnI values with assay 3 were compared to the normalized cTnI values with assays 1 and 6, the cTnI concentrations (mean, SD) in the cTnAAb-positive cohort were not significantly different ($P=0.878$) between cTnI assays 1 (1,820; 2,630 ng/L) and 3 (2,490; 3,270 ng/L), but the concentration with assay 6 (9,190; 14,000 ng/L) was 5.0- and 3.7-fold higher ($P<0.001$) than in the other two assays. In other words, cTnI assay 6 enabled significantly higher cTnI recognition in cTnAAb-positive patient samples.

Further investigation of underlying causes for the differences observed in calculated cTnI concentrations was performed by comparing the relative capture efficiencies of cTnI assays 1 and 6. After preincubation on cTnI assay 6 capture surface, assay 1 detected only 4%-5% of the ITC-derived fluorescence signal while in the reverse situation, assay 6 detected almost 40% of the ITC. Similarly, for cTnI-containing patient samples, assays 1 and 6 detected 16%-31% and 48%-83% of the cTnI, respectively. For the endogenous cTnI, the difference in the recoveries between the assays was much smaller than for ITC demonstrating that these surfaces recognize the standard in a highly different manner. This conceivably stems from the different epitope specificities, affinities and formats of chosen antibodies, all of which also determine the achievable analytical sensitivity for the assay. Thus, the results emphasize the well-known complexity of cTnI assay standardization.

The findings of this substudy established that the cTnAAb-associated interferences are seen in investigational and commercial midfragment targeting cTnI assays. The interferences, however, can be circumvented by using the novel 3+1-type antibody configuration (cTnI assay 6) with three capture antibodies against the N-terminus, midfragment and C-terminus and one tracer antibody against the C-terminus. In essence this means that significant cTnI concentration underestimations are seen in individual cTnAAb-positive samples when using midfragment targeting cTnI assays in relation to this assay. In the light of our results, the risk for clinical misclassification brought up in previous reports remains a valid and reasonable concern (Eriksson *et al.*, 2003; Eriksson *et al.*, 2005b; Eriksson *et al.*, 2005c; Tang *et al.*, 2012).

This substudy was limited to a comparison of measured cTnI values within the ranges considered analytically reliable with all the included assays. Therefore, very low cTnI concentrations could not be investigated and a sensitive assay of the novel 3+1-type design was later developed (see Chapter 5.5). Additionally, not all individual samples behaved similarly with respect to cTnAAb interference; the changed antibody configuration had a stronger effect on the interference in some samples than others. This finding indicated that in addition to cTnAAb titers, the epitope specificities of cTnI-specific autoantibodies can vary between individuals and, thus, affect the degree of the interference observed. Therefore, the fine-specificity of circulating cTnAAb is called for more precise characterization.

5.2 Characterization of cardiac troponin specific auto-antibodies

5.2.1 Epitope specificity

After establishing cTnAAb as an important analytical confounder in state-of-the-art cTnI assays, our purpose in the second substudy (**II**) was to explore more precisely the cTnI epitope specificity and IgG subclass distribution of these autoantibodies. To avoid the problems resulting from the use of spiked TSA-BSA as a reference sample,

the epitope specificity of cTnAAbs was determined by comparing the analytical recoveries in 10 cTnAAb-positive individuals to the recoveries in 10 cTnAAb-negative individuals chosen based on the results obtained with the old cTnAAb assay. The signal recoveries were measured from these 20 apparently healthy subjects with 19 sandwich-type immunoassays using various cTnI-specific antibodies for capturing and the same TnC-specific antibody for detecting.

The mean analytical recovery in cTnAAb-positive samples for all 19 epitopes was 89% whereas the mean recovery values for single cTnI epitopes ranged from 37% to 211% (**Figure 11A**). The lowest mean recoveries were obtained for epitopes on the midfragment and N-terminal parts of the C-terminus. Additionally, considerable variation in individual recoveries of cTnAAb-positive samples was seen: the minimum and maximum recoveries for single epitopes ranged from 4% to 92% and from 78% to 309%, respectively. Although the effects of other sample interferences could not be ruled out from these results, the variation around the mean recoveries (mean value of SDs, SD) was notably lower in cTnAAb-negative individuals (14%, 6%) than in cTnAAb-positive individuals (36%, 19%). Therefore, the comparison between the site-specific recoveries of three cTnAAb-positive subjects shown in **Figure 11B** primarily demonstrated that interindividual variation differs between epitopes.

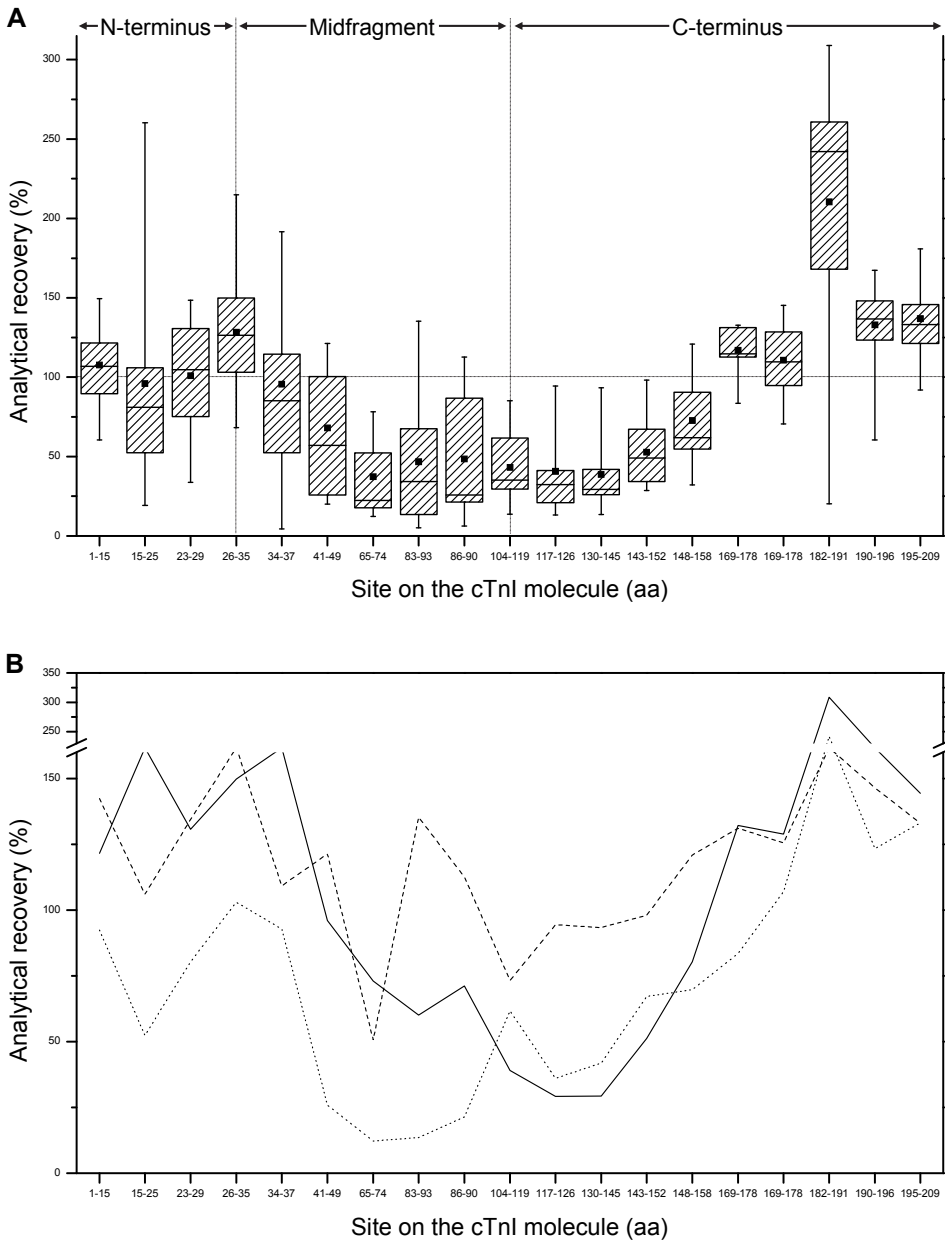


Figure 11. Analytical recoveries for different cTnI sites (adapted from II). A) ITC recoveries in 10 cTnAb-positive samples. Whiskers represent minimum and maximum signals; boxes represent 25th percentile, median, and 75th percentile; and black squares represent means. B) An example of site-specific ITC recoveries in three cTnAb-positive individuals.

The analytical recovery tests of this substudy confirmed the previously published observation (Eriksson *et al.*, 2004) that the midfragment of the cTnI molecule is most frequently targeted by cTnAAb but also demonstrated that cTnAAb interference extends to the flanking termini, especially towards the C-terminus. Although some cTnI epitopes may be less prone to cTnAAb interference, the results showed that none of the studied sites completely escape it and that the area most affected by the interference may vary between different individuals. In addition to being able to block the binding of assay antibodies, the observed >100% recoveries implied that cTnAAb may also stabilize the analyte or enhance the affinity of assay antibodies against their binding sites. However, the extremely high recoveries in this substudy may be related to the recognition of the cTnI-TnC complex, which is naturally susceptible to conformational changes. Furthermore, these findings explained why low cTnAAb interference is seen with the novel 3+1-type cTnI assay design using four carefully selected epitopes, and verified our preliminary assumption that the cTnI-specific interference is even more heterogeneous than initially reported.

5.2.2 IgG subclass distribution

In the second part of publication II, admission and 3-month follow-up samples from 28 NSTE-ACS patients from a substudy (Pettersson *et al.*, 2009) of 81 patients with known cTnAAb statuses determined using the old cTnAAb assay were analyzed with total IgG assay detecting all IgG subclasses and four subclass-specific assays detecting IgG subclasses 1-4 exclusively. Based on the total IgG results, the patients were categorized into two study groups: the first group included 14 cTnAAb-negative patients, and the second group 14 cTnAAb-positive patients at 3-month follow-up.

As illustrated in **Table 10**, all 14 patients selected for the cTnAAb-negative group were determined cTnAAb-negative by the total IgG assay at both sampling points. In the 14 patients selected for the cTnAAb-positive groups, six were positive at admission and eight turned positive at follow-up using the total IgG assay. The specific signals of these IgG-positive samples ranged from 105 to 69,502 counts (median 2,111 counts, 25th-75th percentiles 881-14,838 counts). None of the samples in the cTnAAb-negative group were IgG1 and IgG2 positive, but four admission and five follow-up samples were weakly positive for IgG3 and/or IgG4 (specific signal 125-794 counts, median 242 counts, 25th-75th percentiles 129-334 counts). Although all IgG subclasses 1-4 were observed in the cTnAAb-positive group (specific signals 101-115,579 counts, median 620 counts, 25th-75th percentiles 274-6,247 counts), IgG4 had the highest prevalence and was detected in eight admission and 11 follow-up samples. Additionally, three admission and 10 follow-up samples were positive simultaneously for 2-4 subclasses while seven admission and four follow-up samples were positive only for one subclass. The specific signals were also generally higher at follow-up compared to admission levels indicating higher cTnAAb titers and/or improved binding affinity (**Figure 12**). Differences in specific signal levels between sampling points were statistically significant ($P<0.05$) with all assays except the IgG3-specific assay ($P=0.249$).

Summary of Results and Discussion

Table 10. Prevalence (n) of cTnAAb-positive samples with total IgG and four subclass-specific assays in two study groups at admission and 3 months' follow-up.

Assay specificity	cTnAAb-negative (n=14)		cTnAAb-positive (n=14)	
	Admission	3 months	Admission	3 months
Total IgG	0	0	6	14
IgG1	0	0	1	9
IgG2	0	0	3	6
IgG3	0	4	2	5
IgG4	4	3	8	11

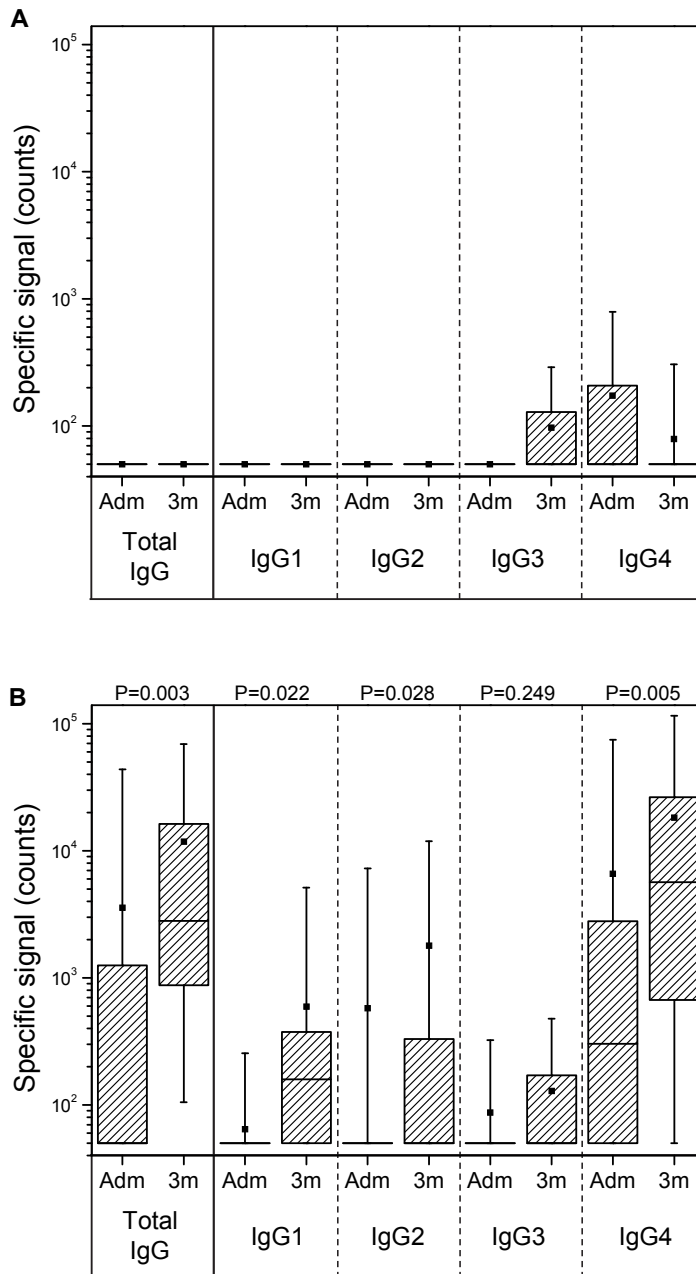


Figure 12. cTnAAb signals in admission (Adm) and 3-month follow-up (3m) samples from 14 cTnAAb-negative (A) and 14 cTnAAb-positive (B) NSTE-ACS patients (adapted from II). The cTnAAb-negative samples have been given a specific signal of 50 counts (the lowest positive signal divided by two). Whiskers represent minimum and maximum signals; boxes represent 25th percentile, median and 75th percentile; and black squares represent means.

Because some patients of the cTnAAb-positive group became positive between sampling points and specific signals with all five IgG assays were generally higher three months after the index event, the results confirmed that cTn leakage from cardiomyocytes during a primary ischemic injury may induce an autoimmune response leading to cTnAAb formation (Leuschner *et al.*, 2008; Pettersson *et al.*, 2009; Lindahl *et al.*, 2010). Alternatively, the leakage may serve as a booster of previous immunization, which can both increase cTnAAb titers in circulation and improve the affinity of formed autoantibodies. However, due to the way the two study groups were compiled, the cTnAAb-positive group represented patients predisposed to cTnAAb formation and thus the observations do not mean that a cTnI leakage alone is sufficient to initiate cTn-related autoimmune responses.

In the cTnAAb-positive group, all IgG subclasses were detected and 46% of individual samples were positive for multiple subclasses, which in turn further increases the heterogeneity of human cTnAABs. Because the frequency of cTnAAb-positive samples observed with certain IgG assays is related to assay sensitivity, which cannot be determined in autoantibody assays due to the lack of defined standards, direct comparison between these frequencies was not possible and the results were partly discrepant. However, it was noteworthy that IgG4, which is generally the least frequent of the four IgG subclasses, was the most commonly detected cTnAAb in this substudy. Because repeated antigenic stimulation is known to promote IgG4-type antibody formation, the appearance of IgG4-type cTnAABs suggested that longer-term cTn leakage may have occurred in cardiac patients before the acute event. This finding is in accordance with the current belief that low levels of circulating cTn are found in virtually all individuals. It is also interesting that IgG4 is generally considered to be a benign and nonpathogenic antibody with positive effects in some allergic reactions (Nirula *et al.*, 2011). Another feature of IgG4-type antibodies is that they may have a high affinity for various animal IgGs, such as mouse IgG, via their constant regions rather than their antigen-binding sites (Kawa *et al.*, 2008; Ito *et al.*, 2010). If IgG4-type cTnAABs bind to animal IgGs used as immunoassay reagents, then in addition to blocking specific cTn epitopes, they could potentially interfere with cTn immunoassays nonspecifically.

5.3 Improved immunoassay for cardiac troponin specific autoantibodies

The methodology used in all our cTnAAb assays has been specifically developed for the recognition of IgG-type antibodies. Therefore, IgM-type antibodies that are predominant in primary immune responses were not studied within the scope of this thesis. Additionally, because ITC is used as a target molecule, the results obtained with the assays do not differentiate cTnI- and cTnT-specific autoantibody positivity whereas error due to nonspecific binding of other human antibodies is taken into account. Sample-specific backgrounds fluctuate substantially between individuals (Eriksson *et al.*, 2005a) and when high, they may limit cTnAAb detection. Therefore,

the main purpose of the cTnAAb assay update described in publication **III** was to reduce the occasionally spurious sample backgrounds. The approach selected was to use alternative capture antibodies to immobilize the cTn-bound autoantibodies. Based on the previous epitope specificity results, we chose the new assay capture epitopes that were less affected by cTnAABs than the epitopes of the old assay (see Chapter 5.2.1). On the other hand, this may at the same time provide detection of cTnAABs of different fine specificity. The method validation was performed by using the old assay as a reference.

Sample-specific backgrounds (n=510) were significantly lower for the new cTnAAb assay (median 1,225 counts, 25th-75th percentiles 973-1,635 counts) than for the old assay (median 2,693 counts, 25th-75th percentiles 2,104-4,043 counts) (P<0.001). When backgrounds from the same samples were compared, the new assay gave on average 29% lower signals than the old assay. In addition, net signals of cTnAAb-positive samples (n=35) were significantly higher for the new assay (median 5,076 counts, 25th-75th percentiles 1,953-17,754 counts) than for the old assay (median 3,921 counts, 25th-75th percentiles 1,326-11,909 counts) (P<0.001). On average, the signals from the same samples were 40% higher for the new assay than for the old assay.

Because of the lower backgrounds and higher signal levels, the new cTnAAb assay described in this substudy was more sensitive than the old cTnAAb assay. Due to the increased sensitivity and/or the different epitope specificity of the used cTnI capture antibodies, the new assay detected 27% more cTnAAb-positive patients than the old assay (P=0.013) while the overall concordance between the two assays was good. From 510 suspected ACS patients, the new assay detected 12 new cTnAAb-positive individuals whose net signals were 103-448 counts. However, two patients who were weakly positive with the old cTnAAb assay (net signals 166-203) were not detected with the new assay. This discrepancy may have been a result of the low cTnAAb titers and/or affinities of these samples because low specific signals do not necessarily differ statistically enough from background signals as the signals slightly vary from run to run. The discrepancy may also have originated from the above mentioned differences in the fine specificities of the two cTnAAb assays.

5.4 Autoantibody prevalence in a heterogeneous patient cohort with suspected myocardial infarction

The prevalence of cTnAABs had not been previously evaluated in a clinically relevant patient cohort where cTn assays are typically applied. Therefore, in publication **III** we determined the prevalence of cTnAABs in a heterogeneous cohort formed by 510 consecutive ACS patients presenting to an emergency department with suspected MI in parallel with the validation of the new cTnAAb assay. We studied the association between the observed cTnAAb status and the previous cardiac conditions possibly leading to cTn leakage, and the association between the observed cTnAAb status and

the outcome in 12 months. The baseline characteristics of the patients are described in detail in the original publication **III**.

The cTnAAb prevalences in both cTnAAb assays in the different diagnostics groups are presented in **Table 11**. Of the whole study cohort, 7% and 9% of patients were cTnAAb-positive with the old and new assay, respectively. For each assay, no statistically significant differences in the prevalences were identified between MI and other patients, or STEMI and NSTEMI patients. The overall prevalences of this substudy were in accordance with the previous reports presented in the literature review (3%-15% in similar cohorts, see Chapter 2.4.1), and demonstrated that approximately one in ten patients who present to a hospital with suspected MI have detectable amounts of cTnAAb in their circulation. The high prevalence of cTnAAb emphasizes the need for larger scale studies regarding the impact of cTnAAb on cTnI testing.

Table 11. Prevalences of cTnAAb in different diagnostic groups with the old and new cTnAAb assay.

Patients	cTnAAb-positive with the old assay	cTnAAb-positive with the new assay
	n (%)	n (%)
All (n=510)	37 (7.3)	47 (9.2)
Non-MI (n=343)	26 (7.6)	32 (9.3)
MI (n=167)	11 (6.6)	15 (9.0)
STEMI (n=70)	3 (4.3)	5 (7.1)
NSTEMI (n=97)	8 (8.2)	10 (10.3)

In this study population, the history of previous cardiac conditions (hypertension, CAD, MI, revascularization and heart failure) was not associated with the presence of cTnAAb, and the presence of cTnAAb did not correlate with the 12-month outcome (reinfarction, revascularization, heart failure and all-cause mortality) (results presented in publication **III**). Thus, this substudy did not provide new information about the reasons for the initiation of cTnAAb-related autoimmune responses or their clinical significance. Larger cohorts are needed to reliably study these associations in patients who develop cTn autoimmunity.

5.5 Sensitive cardiac troponin I assay free from cardiac troponin specific autoantibody interference

The Universal Definition of MI emphasizes the need for reliable detection of minor cTn elevations and changes in these. This calls for effective measures to reduce the false positive or negative effects due to preanalytical and analytical interferences in cTn assays. As cTnAAb can have a decidedly inhibiting effect on cTnI detection, their effects at lower cTnI concentrations presently reachable by contemporary-sensitive and high-sensitivity assays need to be investigated. To enable such a study, we developed in publication **IV** a sensitive cTnI assay free from cTnAAb interference.

The new assay was based on the previously presented assay design employing three separate capture antibodies for the N-terminus, midfragment and C-terminus and one tracer antibody for the C-terminus, on the epitope shown to be virtually unaffected by cTnAAb. Antibody-coated spots were used to achieve higher analytical sensitivity.

The calibration curve ($y=79.28x$) of the developed cTnI assay 7 is presented in **Figure 13A**. The curve was linear up to 50,000 ng/L ($R^2=0.992$) and no high-dose hook effect was seen even with 1,000,000 ng/L. Dilution linearity of endogenous cTnI was evaluated with serially diluted samples (255-53,639 ng/L cTnI) by linear regression analysis, and the assay was linear ($R^2=0.980-0.996$) throughout the measured cTnI range (7.0-53,693 ng/L). Using the CLSI criteria, the LoB and LoD of the assay were 1.4 and 2.9 ng/L, respectively. Total imprecision was determined by running sample pools ($n=8$, 1.0-732 ng/L cTnI) in triplicate once a day for 20 days (**Figure 13B**) using two different spotting batches. The ideal accuracy goal of 10% CV was not reached but with both batches, 20% CV was achieved at 10 ng/L. The assays with total precisions up to 20% at the 99th percentile of healthy population are perfectly usable for MI diagnosis (Apple *et al.*, 2005; Kupchak *et al.*, 2006; Jaffe and Apple, 2010). The high variability of the cTnI assay 7 was presumably caused by the non-uniformity of the antibody-coated spots. Large scale production with optimized spotting techniques in combination with complete automation of the assay protocol would likely result in substantially improved assay precision.

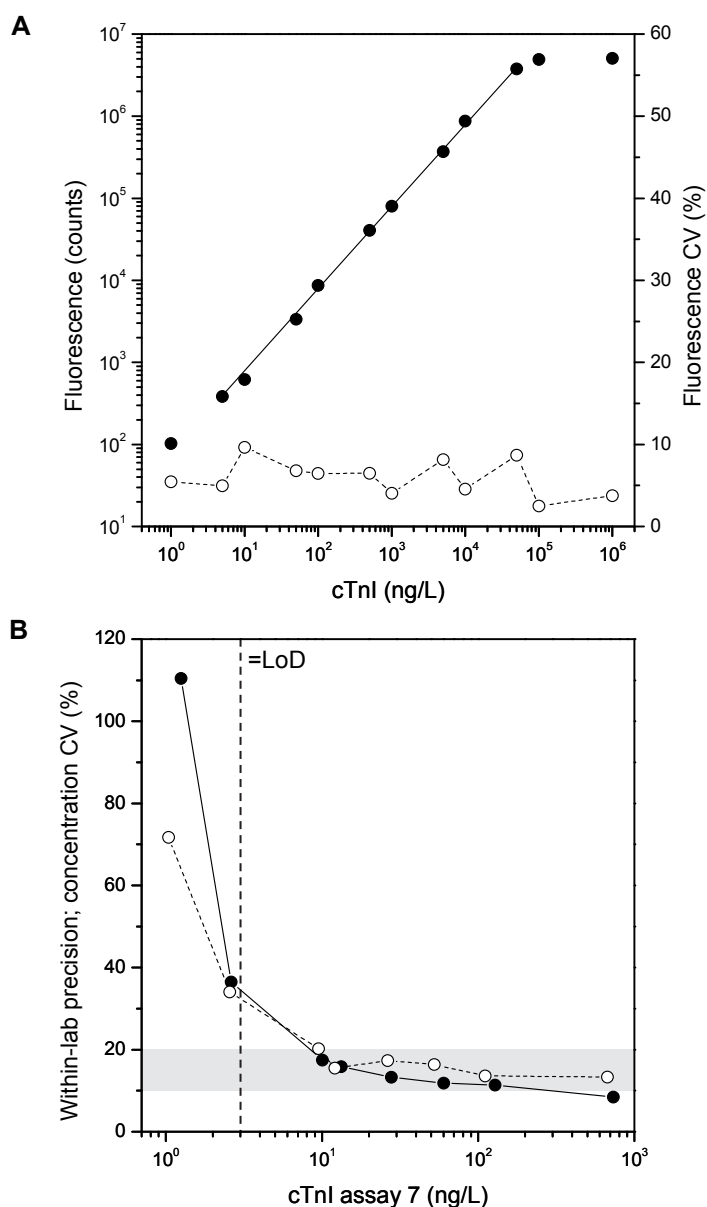


Figure 13. Calibration curve and total precision of cTnI assay 7 (adapted from IV). A) Calibration curve (●) and within-assay precision profile (○) illustrate the means of six replicate reaction wells. B) Total precision profiles for two spotting batches (● and ○) were measured with eight cTnI sample pools.

The resistance to cTnAAb interference of cTnI assay 7 was ascertained with the small-scale recovery study over a wide range of cTnI concentrations in comparison with the midfragment targeting cTnI assay 3 (**Figure 14**). Increasing amounts of ITC

(10-50,000 ng/L cTnI) were added into five samples, two of which were cTnAAb-negative and three cTnAAb-positive with the new cTnAAb assay. In the two cTnAAb-negative samples, both assays showed good recoveries over the whole measurement range. In the three cTnAAb-positive samples measured with cTn assay 3, ITC amounts up to 1,000 ng/L remained below the analytical sensitivity of the assay (60 ng/L). Individual recoveries with each of these samples increased from the smallest to the highest detectable analyte concentration and the mean recovery was 28% while being 85% for the cTnAAb-negative samples. In the three cTnAAb-positive samples measured with cTnI assay 7, all ITC amounts added were detectable and recoveries were high over the studied range. The mean recoveries were 100% and 119% for the cTnAAb-positive and cTnAAb-negative samples, respectively. The comparison demonstrated that in cTnAAb-positive samples, cTnI levels can be seriously underestimated with state-of-the-art assays affected by cTnAAb interference to the extent that the early cTnI release is not recognized. A repeated measurement 3-6 h after admission may conceivably pick up the ACS-related cTnI increase but in some cTnAAb-positive samples, even high cTnI concentrations can be severely blunted.

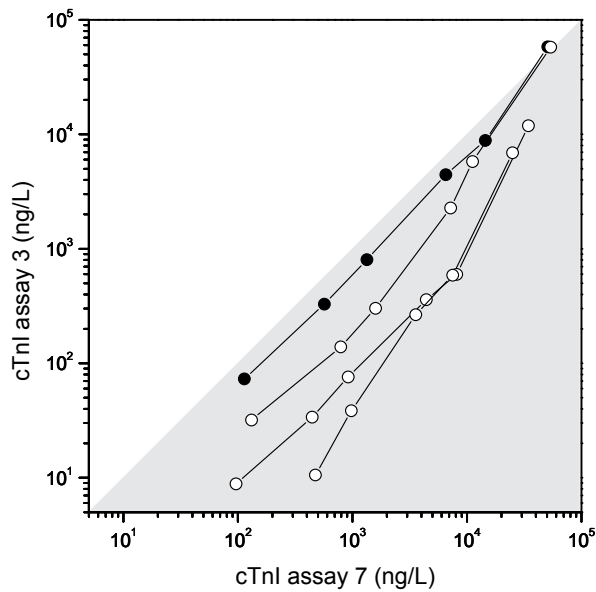


Figure 14. Means of measured cTnI values in two ITC-spiked cTnAAb-negative individuals (●), and measured cTnI values separately in three ITC-spiked cTnAAb-positive individuals (○) (adapted from IV).

Because the tracer antibodies used in our 3+1-type assay designs bind to the C-terminus of the cTnI molecule, the more stable midfragment alone is not recognized. Therefore, the stability of endogenous cTnI after drawing blood was studied with cTnI assay 7 using 10 samples containing 16-26,158 ng/L cTnI. After 1, 3 and 5 freeze-

thaw cycle(s), the median (25th-75th percentiles) recoveries from the original cTnI values were 104% (89%-123%), 101% (65%-118%) and 107% (79%-130%), respectively. The corresponding values after incubating the samples for one day or one week at +4°C were 103% (83%-117%) and 102% (77%-123%), and at room temperature 102% (77%-109%) and 71% (61%-98%), respectively. Differences between the studied conditions were not statistically significant ($P=0.051$) demonstrating surprisingly good analyte stability. Nevertheless, longer storage at room temperature seemed to decrease the detected cTnI concentrations. One limitation of this stability study was that we did not have fresh samples to include in the study. However, when compared to freshly analyzed samples, only minor losses of immunoreactivity of clinical samples incubated for 24h at room temperature were previously reported with 2+1-type cTnI assay (Radiometer) also unable to generate signal from the midfragment (Eriksson *et al.*, 2005c; Hedberg *et al.*, 2006).

Of 250 patients analyzed for the method comparison, 34 (14%) were cTnAAb-positive with the new cTnAAb assay. Because cTnI assay 7 was expected to suffer less from cTnAAb interference than the midfragment targeting Architect hs-cTnI assay, the comparison was limited to cTnAAb-negative samples with cTnI concentrations exceeding the LoDs of both assays ($n=160$). Although the correlation was good (Spearman $r=0.958$, $P<0.001$), a considerable systemic bias was seen between the absolute cTnI concentrations measured with the two assays; Architect hs-cTnI assay gave on average 7-fold higher cTnI concentrations than the new assay. Deming regression yielded a slope (95% confidence intervals) of 0.20 (0.17-0.22) and y-intercept of 1.65 (0.78-2.52) ng/L ($S_{y|x}=0.21$ ng/L) (**Figure 15A**). The mean relative difference (95% limits of agreement) with Bland-Altman agreement was 134% (70%-198%) (**Figure 15B**). The systemic bias conceivably stems partly from the use of different antibodies but also from the differences in calibration and standard material (our cTnI standards 5; 50; 500; 5,000 and 50,000 ng/L gave with Architect hs-cTnI only 3; 16; 154; 1,820 and 30,169 ng/L, respectively, **unpublished data**). Similar biases have been previously obtained by comparing the above mentioned 2+1-type cTnI assay to the midfragment targeting AxSYM first-generation cTnI assay and the midfragment targeting AccuTnI second-generation assay (Hedberg *et al.*, 2006) but also by comparing the midfragment targeting Liaison cTnI assay to AccuTnI second-generation assay (Pagani *et al.*, 2004). Again, the observed difference highlights the complexity of cTnI standardization. The relative difference in the method comparison study was reasonably constant over the whole cTnI range.

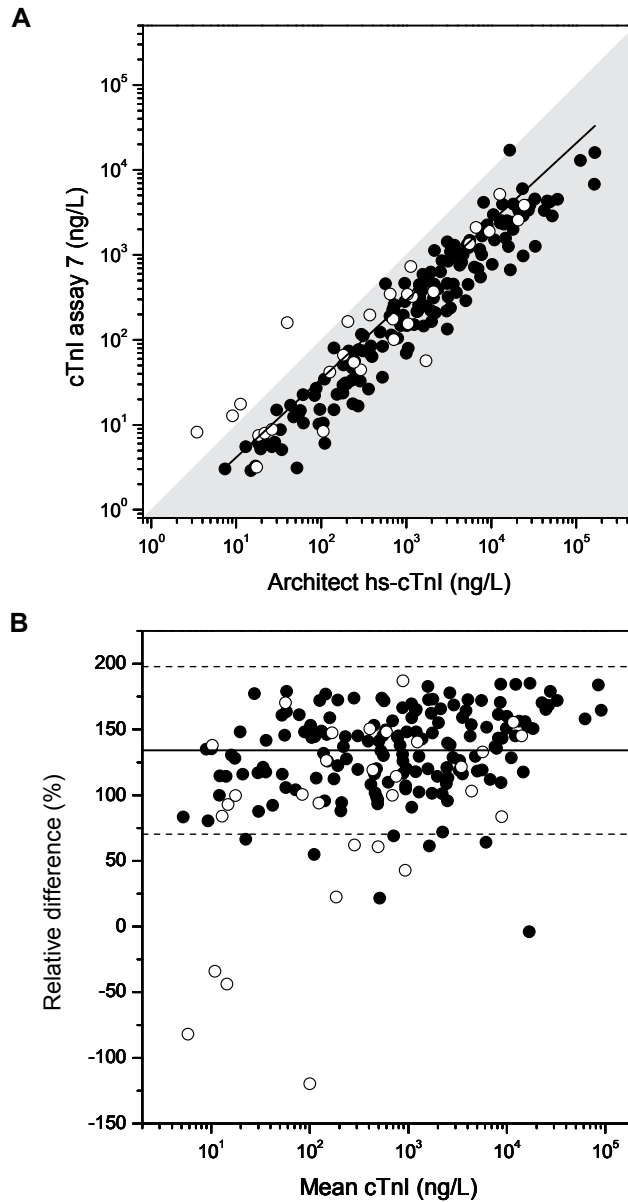


Figure 15. Method comparison with cTnAAb-negative (●) and cTnAAb-positive (○) samples (adapted from IV). A) With cTnAAb-negative samples, Deming regression equation (solid line) was $y=0.20x+1.65$. B) With cTnAAb-negative samples, the mean difference [(Architect hs-cTnI - cTnI assay 7) / mean cTnI] was 134% (solid line) and 95% limits of agreement were 70% and 198% (dashed lines).

Although the method comparison was limited to the cTnAAb negative samples, the results from those cTnAAb-positive samples with cTnI concentrations above the LoDs of both assays (n=30) were included in **Figure 15**. Four of the samples with low cTnI values clearly deviated from the scatter of the cTnAAb-negative samples because of the higher cTnI values with cTnI assay 7 than with Architect hs-cTnI assay which implies that cTnAAbs might have an impact on cTnI testing. However, the small number of cTnAAb-positive samples whose cTnI values were at the reliable ranges of both assays prevented making any final conclusions. Significantly larger patient cohorts would be needed to address this question.

The reference population included 159 apparently healthy individuals (50% females). The samples had been grouped so that there were 65 samples from individuals 31-50 years of age, 47 samples from individuals 51-70 years of age, and 47 samples from individuals >70 years of age. Of all individuals, 28 (18%) had a measured cTnI concentration above the LoD of cTnI assay 7 and 16 (10%) were cTnAAb-positive with the new cTnAAb assay (**Figure 16**). As previously mentioned, the recent criteria for reference interval studies recommends that a minimum number of 300 individuals are needed to appropriately determine the 99th percentile and more thorough screening (e.g. with ECG) of sample donors is warranted to detect any underlying cardiac conditions (Apple *et al.*, 2012a; Sandoval and Apple, 2013). Therefore, due to the small number of apparently healthy individuals and their poor clinical characterization, we refrained from 99th percentile calculations. In addition, the developed cTnI assay did not meet the goal proportion of 50% measurable from a healthy reference population; therefore, it could not be classified as a high-sensitivity assay. Yet, the proportion of analytically reliable concentrations with cTnI assay 7 was comparable to the most sensitive contemporary and point-of-care assays; in a recent publication, the proportion was more than 6% only for one contemporary and one point-of-care cTnI assay (Apple *et al.*, 2012b).

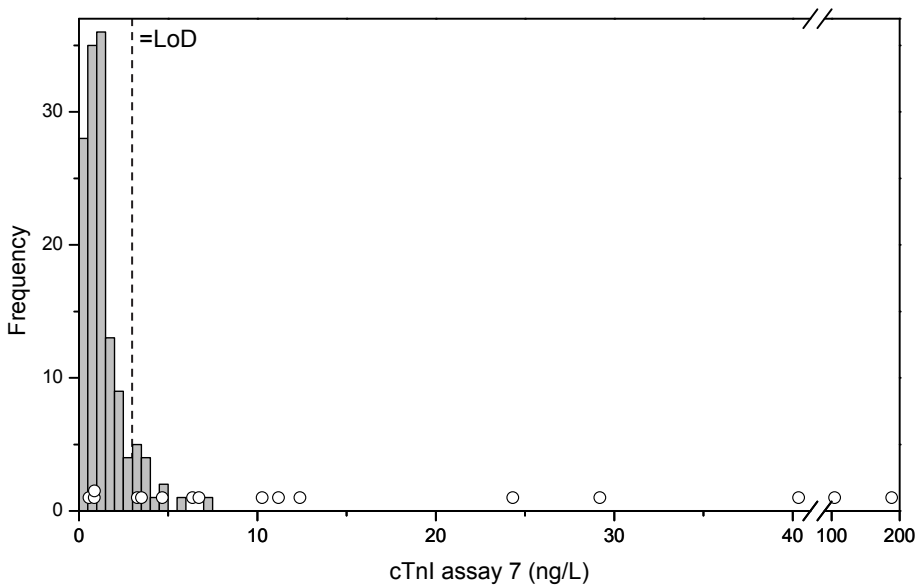


Figure 16. Distribution of measured cTnI values in apparently healthy individuals (n=159) (adapted from **IV**). The circulating cTnI values were higher in cTnAAb-positive (circles) than in cTnAAb-negative (columns) samples (median 8.5 ng/L vs. <LoD).

Interestingly, the proportion of measurable cTnI values was significantly higher in the cTnAAb-positive group (13/16) than in the cTnAAb-negative group (15/143) ($P < 0.001$). The median of measured cTnI concentrations (25th-75th percentiles) for the cTnAAb-positive group was 8.5 (3.4-28) ng/L, the highest concentrations reaching up to 100-200 ng/L, whereas the median for the cTnAAb-negative groups was <LoD. Although the impact of circulating cTnAAbs on the patient outcome has remained controversial (Shmilovich *et al.*, 2007; Leuschner *et al.*, 2008; Miettinen *et al.*, 2008; Dungen *et al.*, 2010; Lindahl *et al.*, 2010; Doesch *et al.*, 2011), the presence of cTnAAbs has been associated with chronically elevated cTnI concentrations (Plebani *et al.*, 2002; Pettersson *et al.*, 2009; Lindahl *et al.*, 2010) and persistent cTnI elevations in turn with higher mortality during long-term follow-up (Eggers *et al.*, 2007). Therefore, the high cTnI concentrations in apparently healthy cTnAAb-positive individuals raise new questions about the etiology of cTnAAbs. The higher concentrations may result from a cTnAAb-associated persistent injury or reflect a longer half-life on circulating cTnI-cTnAAb complexes.

Mab 8I7 used in cTnI assay 7 as a tracer was recently reported to recognize skTnIs (Vylegzhanina *et al.*, 2013). Therefore, employing it for cTnI detection together with antibodies lacking cTnI-specificity could result in falsely high cTnI values in cases of increased skTnI. Alternatively, when Mab 8I7 is paired with antibodies having high cTnI-specificity, increased skTnI concentration could result in falsely low cTnI values

due to the competition between cTnI and skTnI for 8I7 binding. Although our preliminary data with cTnI assay 7 indicates no such problems, this warrants for further investigation.

Although the developed cTnI assay facilitates sensitive and reliable cTnI detection in the presence of circulating cTnAAbs, the assay is not suitable for routine clinical practice in its present form because of the long assay time. The presented 3+1-type assay must be developed further to enable fast and automated cTnI detection while maintaining the achieved analytical sensitivity or even improving it.

6 CONCLUSIONS

Due to their unique specificity and sensitivity for myocardial injury, cTnI and cTnT are the recommended biomarkers for the diagnosis and risk stratification of patients with suspected ACS. Since the introduction of the first immunoassays for cTn detection towards the end of the 1980s and the beginning of the 1990s, the analytical sensitivities and precisions have progressively improved. This course has been driven by the first Universal Definition of MI that challenged assay manufacturers to develop better cTn assays to reach the total precision of $\leq 10\%$ at the 99th percentile cutoff value determined from healthy individuals. The newest generation of sensitive-contemporary and high-sensitivity cTn assays has recently enabled the measurement of cTn levels in healthy individuals and, thus, more rapid and accurate detection of MI. Because low cTn values achievable with these assays are inherently more susceptible to various analytical confounders, it is important that the low-end accuracy of the assays is ascertained by minimizing preanalytical and analytical problems leading to false-positive and false-negative results.

Circulating cTnAAb that are commonly found in individuals with or without cardiac diseases can negatively interfere with cTn detection using cTnI immunoassays designed according to the IFCC-recommended midfragment approach, to the extent that cTnAAb-positive patients may be falsely designated as cTnI-negative. In order to establish the clinical impact of these autoantibodies, new immunoassays for cTnI and cTnAAb determination were developed based on the cTnAAb characteristics discovered during this study.

The main conclusions based on the original publications are presented below:

- I** The analytical recovery tests and additional studies with NSTEMI-ACS patient samples demonstrated the notable interference of circulating cTnAAb in representative midfragment targeting cTnI assays used in clinical practice. This particular analytical interference can be avoided by using the novel 3+1-type cTnI assay design with three capture antibodies against the N-terminus, midfragment and C-terminus and one tracer antibody against the C-terminus.
- II** The epitope specificity and IgG subclass distribution of cTnAAb demonstrated that these autoantibodies are extremely heterogeneous. The midfragment of cTnI is most frequently targeted by cTnAAb but the interference extends to the flanking termini, encompassing basically the whole cTnI sequence. There seems to be also remarkable individual variation at the affected sites. Thus, it is not surprising that multiple assay antibodies are needed to circumvent the cTnAAb-associated interferences in cTnI detection. Furthermore, the IgG substudy showed that cTnAAb formation may be triggered or boosted in acute cardiac events.

- III In this publication, an improved version of an existing cTnAAb assay was developed. The new cTnAAb assay detected more cTnAAb-positive patients than the old cTnAAb assay, yet with good overall concordance between the assays. Moreover, nearly one in 10 patients who presented to the emergency department with suspected ACS had cTnAAbs in their circulation which could not be explained by the history of previous heart conditions.
- IV A new sensitive cTnI assay based on the above presented 3+1-type assay design and antibody-coated spots was developed. It enables reliable cTnI detection in the presence of cTnAAbs even at low cTnI concentrations detectable by the commercial cTnI assays at clinical use.

In conclusion, the findings of this thesis showed that circulating cTnAAbs are common in patients with suspected ACS and that cTnAAbs can inhibit cTn detection when targeted against the binding sites of antibodies used in its immunological detection. Therefore, the risk of clinical misclassification due to cTnAAbs remains a valid and reasonable concern. As the impact of cTnAAbs on cTnI testing is multifaceted, i.e. affected by the titers, affinities and epitope specificities of these highly heterogeneous autoantibodies and by the concentration of endogenous cTnI, significantly larger patient cohorts are needed to establish the frequency of cTnAAb-related erroneous cTnI results that may have an effect on patient management. In this the developed cTnI and cTnAAb assays could serve as important analytical tools. Results from these studies could then be used to address the question whether the analytical effect of cTnAAb needs to be acknowledged in the design of future cTnI assays. And more specifically, if common assay antibodies will be selected for all cTnI assays, it is presumable that compromises between various analytical confounders have to be made and thus objective data to support the final decisions are of great importance. Furthermore, the new assay can help unravel the etiology of cTn-related autoimmune responses and their clinical significance.

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